Stereospecific bioanalysis of ibuprofen and flurbiprofen : application to dispositional studies in humans.

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STEREOSPECIFIC BIOANALYSIS OF IBUPROFEN AND FLURBIPROFEN : APPLICATION TO DISPOSITIONAL STUDIES IN HUMANS

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“ It isn’t that they can’t see the solution.

It is that they can’t see the problem.”

G.K. Chesterton 1874-1936:
The Scandal of Father Brown (1935)

ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are known to be the major class of agents responsible for adverse effects in the elderly population. Age-related alterations in drug disposition are well established and are probably a contributory factor to adverse reactions in the elderly. However, the majority of NSAIDs are used as racemic mixtures and little is known concerning the influence of ageing on the stereochemical aspects of drug disposition. These studies were therefore initiated to investigate the stereoselective disposition of ibuprofen and flurbiprofen in young and elderly volunteers. Although, both of these drugs are members of the 2-arylpropionic acid family of NSAIDs, they display considerable differences in their stereoselective disposition and pharmacodynamics.

Enantiospecific analysis of ibuprofen in serum and urine was based on the indirect approach to chiral chromatography, involving derivatization with (*R*)-1-(naphthen-1-yl) ethylamine to yield diastereomeric amides followed by reversed-phase HPLC. Protein binding of the individual enantiomers was performed using equilibrium dialysis followed by direct resolution using a derivatized cellulose CSP (Chiracel OD). Determination of the stereochemical composition of its major metabolites, hydroxy- and carboxyibuprofen, in urine was carried out by sequential achiral-chiral chromatography using a derivatized amylose CSP (Chiralpak AD). Simultaneous enantiospecific analysis of flurbiprofen and its metabolites, 4'-hydroxy- and 3'-hydroxy-4'-methoxyflurbiprofen, in urine was by chiral-phase chromatography using the derivatized amylose CSP. Modification in the mobile phase composition allowed the analysis of flurbiprofen and 4'-hydroxyflurbiprofen in serum. This CSP was also used to isolate sufficient quantities of the individual enantiomers of both flurbiprofen metabolites for chiroptical characterization.

Following the administration of the racemic drugs to both young and elderly volunteers enantioselective disposition was displayed by both compounds, the more prominent enantiomeric differences observed with ibuprofen. The serum kinetics of ibuprofen showed statistically significant differences ($p<0.05$) with (*S*)-ibuprofen showing greater CL, V_d and $t_{1/2,z}$ and unbound fraction in comparison to the *R*-enantiomer in both age groups. Similarly, examination of the urinary composition of ibuprofen and its metabolites showed preferential elimination of products with the *S*-configuration. However with flurbiprofen, the *S*-enantiomer had a significant lower CL,

greater AUC and $t_{1/2,z}$ in comparison to the *R*-enantiomer in the elderly group. Similar trends were observed in the young, although only $t_{1/2,z}$ showed statistical significance. Furthermore urinary excretion of flurbiprofen and its metabolites, in contrast to ibuprofen, displayed a more modest preference for the *R*-enantiomer.

Age-associated alterations in the disposition of ibuprofen were evident for the *S*- and not the *R*-enantiomer. As a consequence of a higher unbound fraction and reduced unbound clearance, which was largely due to reduced clearance via oxidative metabolism, the elderly had a greater exposure to the free levels of the pharmacologically more potent (*S*)-ibuprofen leading to a 25% increase in unbound AUC with age. Pharmacodynamic studies, based on monitoring the inhibition of TXB_2 formation, showed that ibuprofen activity is closely correlated with unbound (*S*)-ibuprofen concentration. Age-related differences in the pharmacokinetics of flurbiprofen seem to stem primarily from a reduction in clearance as a result of a general decline in metabolic activity. Consequently, the elderly have a greater exposure to both enantiomers of the drug with enantiomeric AUCs typically 60% higher in this age group in comparison to the young. These age-related alterations in the disposition of ibuprofen and flurbiprofen may contribute to the adverse reactions in this population sub-group.

ACKNOWLEDGEMENTS

There are a number of people I want to say thanks for all their efforts, both academic and otherwise.

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CHAPTER 1 :

Introduction

1.1. Introduction

Chirality of drug substances has received inadequate attention in the past and we have learned through experience that we ignore this aspect of molecular structure at our peril. In other words, the stereochemical genie has finally escaped from the medical prison in which it has been confined for too long. Advances over the last two decades in the area of stereospecific analysis, with parallel developments in stereoselective synthesis, have provided us with the tools to realise the potential significance of the pharmacodynamic and pharmacokinetic properties of the enantiomers of chiral drugs. This general increase in awareness is reflected in the attitude of regulatory authorities around the world. Although, geometrical isomers have long been regarded as different compounds, it is becoming increasing apparent that regulatory bodies also take this view when dealing with the enantiomers of a chiral drug (Caldwell, 1999). The initial sections in this chapter are designed to provide the reader with a brief overview of stereochemistry and its role in drug action and disposition. As the studies presented in this thesis are concerned with investigating the influence of ageing on the stereoselective disposition of 2-arylpropionic acids, the remainder of this chapter is devoted to a review of the literature on the stereoselective pharmacology and methods of enantiospecific analysis of this important sub-group of non-steroidal anti-inflammatory drugs (NSAIDs).

1.2. Stereochemistry

Stereochemistry is concerned with the three dimensional spatial arrangement of the atoms within a molecule. The prefix stereo originating from the Greek *stereos* meaning solid or volume. The origins of stereochemistry stem from the leap of intuition made by Louis Pasteur in 1849 concerning the "handedness" he noted in crystal structure (Drayer, 1993). By crystallisation of sodium ammonium tartrate from aqueous solutions, he obtained two types of hemihydric crystals with facets arranged such that non-superimposible species were formed. After separating the crystals by hand, he re-dissolved them individually and found that one solution rotated the plane of plane polarised light to the left ((-)-tartrate) and the other to the right ((+)-tartrate). This led

him to the conclusion that optical activity was a characteristic of the molecule and that the individual molecules of (-)- and (+)-tartaric acid are stereochemically dissymmetric, related to each other as non-superimposible mirror images and expressed as hemihydric crystals in their sodium ammonium salt form. The two forms are known as enantiomorphs or enantiomers (Greek *enantios*, opposite; *morph*, form). Compounds which display this behaviour are said to be chiral (Greek *chiros*, handed), because like an individual's hands, the individual stereoisomers are not superimposible with their mirror images (Hutt, 1998).

1.2.1 Enantiomers and diastereoisomers

The chirality of biologically-significant compounds generally arises as a result of the presence of a tetravalent carbon atom in a molecule to which four different atoms or groups are bonded, such atoms are known as centres of asymmetry or chiral centres (Figure 1.1). In addition to carbon, other atoms such as nitrogen, phosphorus and sulphur to which four different groups are bonded also generate chiral molecules of pharmacological importance (Hutt, 1998). The presence of one chiral centre (n=1) in a molecule gives rise to a pair of enantiomers, whereas n such different centres yields 2^n stereoisomers and half as many pairs of enantiomers (Figure 1.1). Those isomers that are not enantiomeric are diastereomeric. It is also noteworthy, that molecules which do not possess an asymmetric centre may still exist in enantiomeric forms as a result of an axis or plane of chirality, however such systems are not often encountered in compounds of pharmacological interest.

In a pair of enantiomers the distances between nonbonded atoms are identical and so enantiomers tend to have identical physiochemical properties except that they rotate the plane of polarised light by equal amounts but in opposite direction. Thus, they have essentially identical melting and boiling points, lipid solubilities, chromatographic characteristics and spectra. The energy differences between enantiomers are so little as to make distinction between them extremely difficult unless they are placed in a chiral environment, and this has profound importance in terms of both pharmacology and analytical chemistry. When two chiral molecules interact, either by the formation of a covalent or ionic bond or other weaker attractive forces, the physiochemical differences between the formed diastereomeric compounds or complexes are considerably more distinct. Diastereoisomers tend to have different energy contents unlike enantiomers,

since distances between nonbonded atoms are not identical, and hence they can be expected to be more easily discriminated by their different chromatographic properties, melting points and spectra.

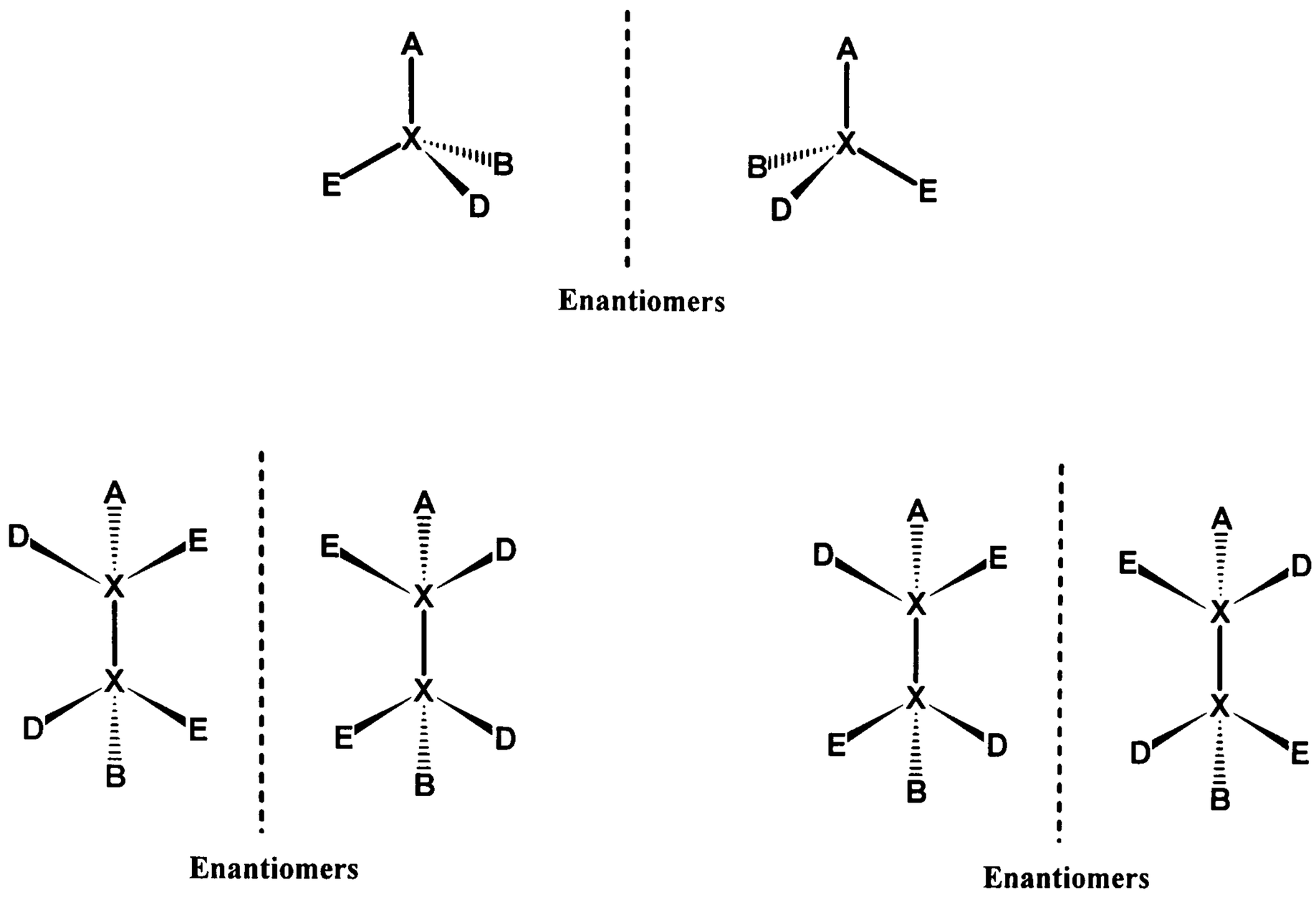


Figure 1.1: Relationship between enantiomers and diastereoisomers (X denotes chiral centre).

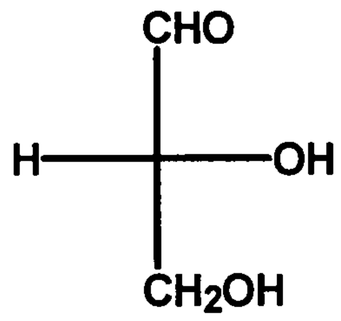
1.2.2 Nomenclature

The traditional approach used to distinguish between a pair of enantiomers relied upon their differential interactions with plane polarised light. Under a particular set of experimental conditions, the isomer which rotates light to the right being termed dextrorotatory (*d*- or (+)-) and conversely, the isomer which rotates light to the left being designated levorotatory (*l*- or (-)-). A racemic mixture, a 1:1 mixture of the enantiomers, being indicated by either *d,l*- or (±)- prefix to the name of the compound. This notation describes a physical property of the molecule and indicates whether a single isomer or mixture is present but does not describe the actual spatial arrangement of the atoms or groups around the chiral centre, i.e. provides no information on its absolute configuration.

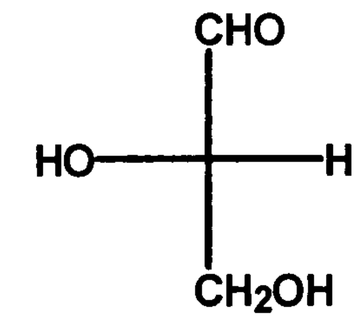
At the end of the 19th century, the means to determine spatial three dimensional arrangement of the atoms or groups within a molecule, i.e. the absolute configuration,

were not available and it was realised that reference standards were required. The carbohydrate, (+)-glyceraldehyde, was arbitrarily assigned the D-configuration when drawn as a Fischer projection (Figure 1.2) and any compound which could be synthesised from (+)-glyceraldehyde without inversion of the chiral centre was, by definition, the D-isomer; conversely, compounds related to (-)-glyceraldehyde were designated the L-isomer. It was not until the 1950s that X-ray crystallographic studies had established that the assigned configuration for (+)-glyceraldehyde was, in fact, correct (Shah *et al.*, 1998). In a similar manner, D-(+)- and L-(-)-serine have also been used for the assignment of relative configurations (Figure 1.2). Due to the difficulty of chemical transformation, the confusion between the lower and upper case notations (D and *d*, L and *l*) and the difficulties in applying the system in many situations, the use of the D,L notation has largely been restricted to defining the stereochemistry of carbohydrates and amino acids.

glyceraldehyde

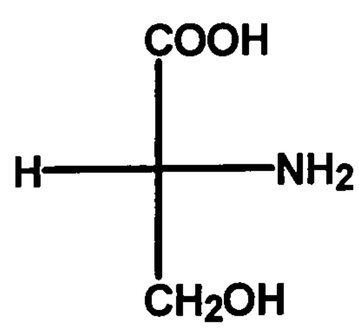


D-(+)-

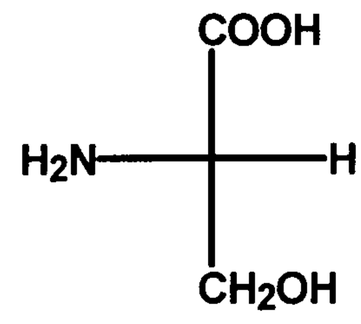


L-(-)-

serine



D-(+)-

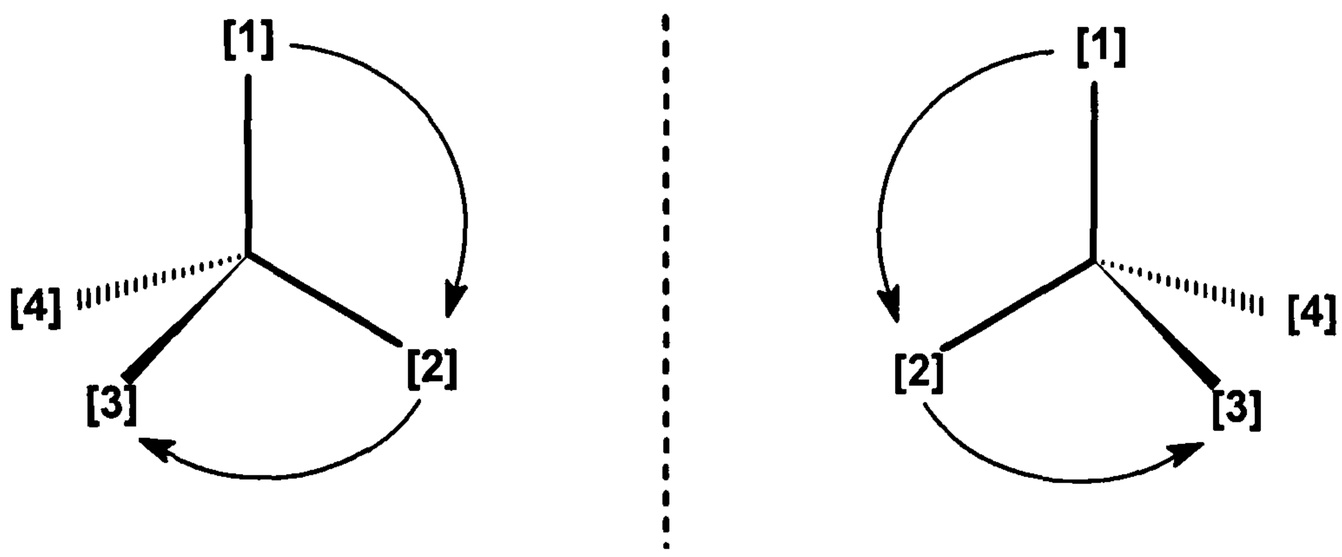


L-(-)-

Figure 1.2: Fischer projections of the enantiomers of glyceraldehyde and serine.

The most widely used system to designate stereoisomers is the sequence rule system, which is also referred to as the Cahn, Ingold and Prelog convention (Cahn *et al.*, 1956). Following establishment of the three dimensional structure of the compound, by techniques such as X-ray crystallography, nuclear magnetic resonance or circular dichroism, the four substituent atoms directly attached to the chiral centre are placed in order of priority according to their atomic number, with the highest atomic number

being assigned the highest priority. If attached atoms are equivalent then the decision on priority is based on the atom an extra bond away and so on until the rank order for the four groups has been established. The molecule under examination is then viewed from the side opposite to the group of lowest priority (Figure 1.3). The arrangement of the remaining three groups is defined as either a *R*- or *S*- absolute configuration, depending on whether the decreasing priority order is clockwise (*R*, rectus) or anticlockwise (*S*, sinister). This convention can be used to rapidly and unambiguously specify the configuration of a chiral centre and is extremely useful for describing diastereoisomers. In the latter case, each chiral centre is designated independently and the configuration of the whole molecule can be easily assigned.



***R*-enantiomer** **S-enantiomer**

Figure 1.3: Assignment of absolute stereochemical configuration according to the Cahn, Ingold and Prelog convention, where the priority order is [1] >[2] > [3] > [4].

1.3. Stereoselectivity in drug action and disposition

1.3.1 Stereoselective pharmacodynamics

Biological systems at a molecular level are homochiral environments with structural, functional and catalytic components composed of biomolecules, e.g proteins, glycolipids and polynucleotides, built from the chiral precursors of L-amino acids and D-carbohydrates. It is therefore not surprising to expect differential interactions between chiral biological macromolecules, such as receptors and enzymes, and the enantiomers of a chiral drug. The first recorded observations of the difference in pharmacodynamic activity of drug enantiomers was attributed to Cushny, who demonstrated that (-)-

hyoscyamine was more potent than the (+)-enantiomer and that (-)-adrenaline had greater activity than its (+)-antipode (Hutt, 1998). According to the Easson and Stedman model (Easson and Stedman, 1933), stereochemical differences in pharmacological activities are due to the differential binding of enantiomers to a common site on an enzyme or receptor surface (Figure 1.4). The model proposed that the more potent enantiomer has a minimum of three intermolecular interactions with the enzyme or receptor surface, whereas the less potent isomer may interact only at two sites. Such a model, although useful, is a rather simplistic representation of a complex bimolecular interaction process. The model assumes the drug molecule has to adopt a particular orientation when interacting with complementary binding sites and can not account for the possible involvement of mutually induced conformational adjustments of the drug and macromolecule. However, a dynamic model of the chiral recognition procedure has been recently proposed by Booth *et al.* (1997), which is based upon molecular chiralities rather than three-point interactions. This so-called "conformation-driven" chiral recognition process involves the initial formation of the complex, followed by conformational adjustment of the two elements, activation of the complex through additional binding interactions and "expression of the molecular chiralities" of the two elements in the complex (Booth *et al.*, 1997).

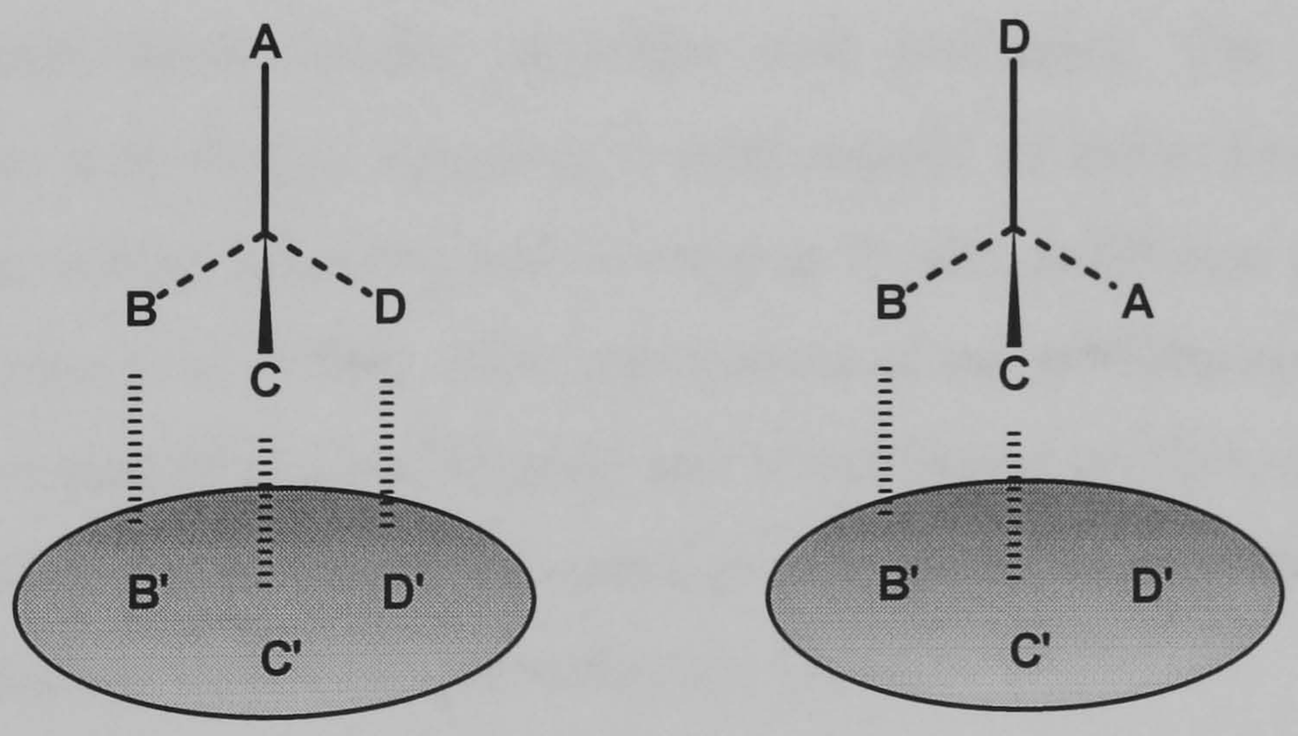


Figure 1.4: Interaction of two enantiomers with a receptor site. The enantiomer on the left is the more potent, with three molecular interactions while that on the right interacts at two points only.

The differential pharmacodynamic activity of a pair of enantiomers has resulted in additional terminology such that the enantiomer with the greater affinity or activity is termed the eutomer and that with the lower affinity or activity is referred to as the distomer (Lehmann *et al.*, 1976). The ratio of their activities, the eudismic ratio is a

measure of the stereoselectivity of the system. It is important to appreciate that the above terms apply to a single drug activity and thus for a dual-action drug, the eutomer for one activity may be the distomer for the other. A variety of differential enantiomer contributions to the biological effects of a drug administered as a racemate are therefore possible and these are outlined below:

Pharmacological activity resides in only one enantiomer. Although highly desirable, it is uncommon for the pharmacological activity to reside in a single enantiomer with the antipode totally devoid of activity. However, one such example is α-methyldopa (Figure 1.5), the antihypertensive activity resides solely in the *S*-enantiomer (Gillespie *et al.*, 1962) and this agent is marketed as a single isomer.

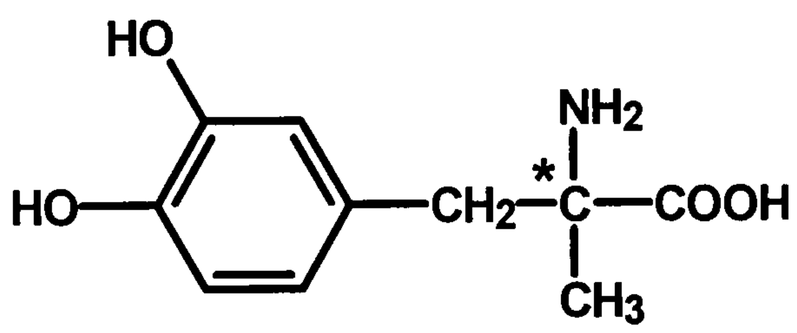
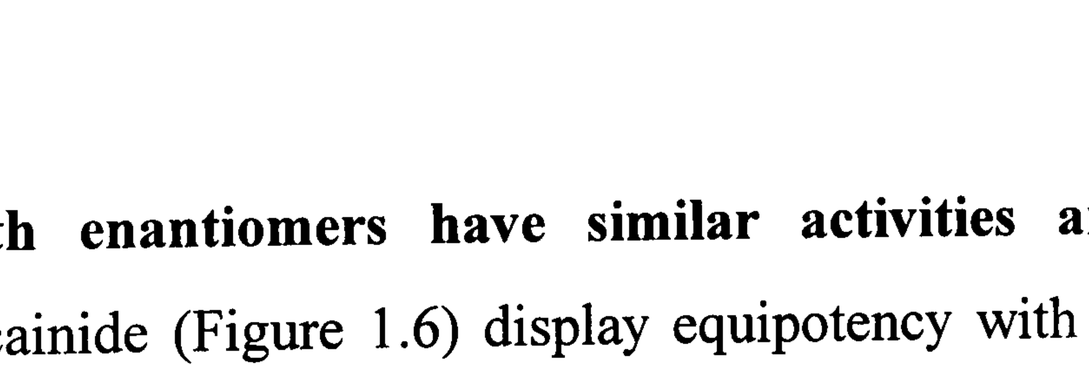
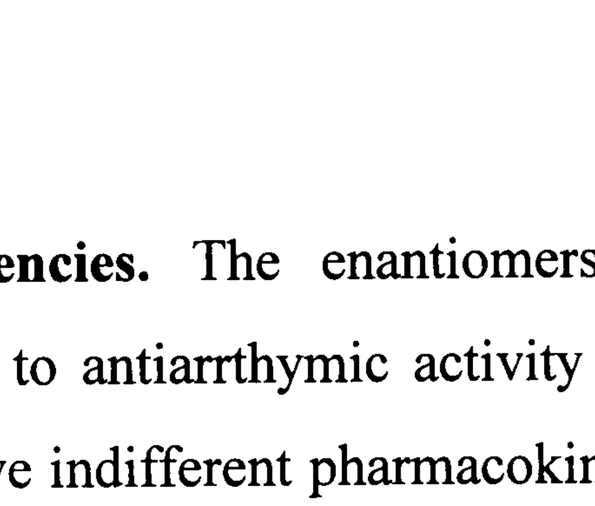


Figure 1.5: Structure of α-methyldopa (* denotes chiral centre).

Both enantiomers have similar activities and potencies. The enantiomers of flecainide (Figure 1.6) display equipotency with regards to antiarrthymic activity and action on cardiac sodium channels; and also appear to have indifferent pharmacokinetic properties (Kroemer *et al.*, 1989). Both enantiomers of the antihistamine promethazine (Figure 1.6) have similar pharmacological and toxicological profiles, and interestingly the loss of chirality in the dimethylaminoethyl side chain is associated with a 50 % decrease in antihistaminic activity (Powell *et al.*, 1988).



(a)



(b)

Figure 1.6: Structure of (a) flecainide and (b) promethazine.

Both enantiomers have similar activities but different potencies. This is the most common situation for racemic drugs. The β-blocking activity of propranolol (Figure 1.7) and other aryloxypropanolamine β-antagonists resides principally in the *S*-enantiomers and the eudismic ratios vary from 100 for propranolol to 10 for atenolol (Walle *et al.*, 1988). Another well known example is warfarin (Figure 1.7), whose *S*-enantiomer is two to five fold more active as an anticoagulant than its *R*-antipode (O' Reilly, 1974).

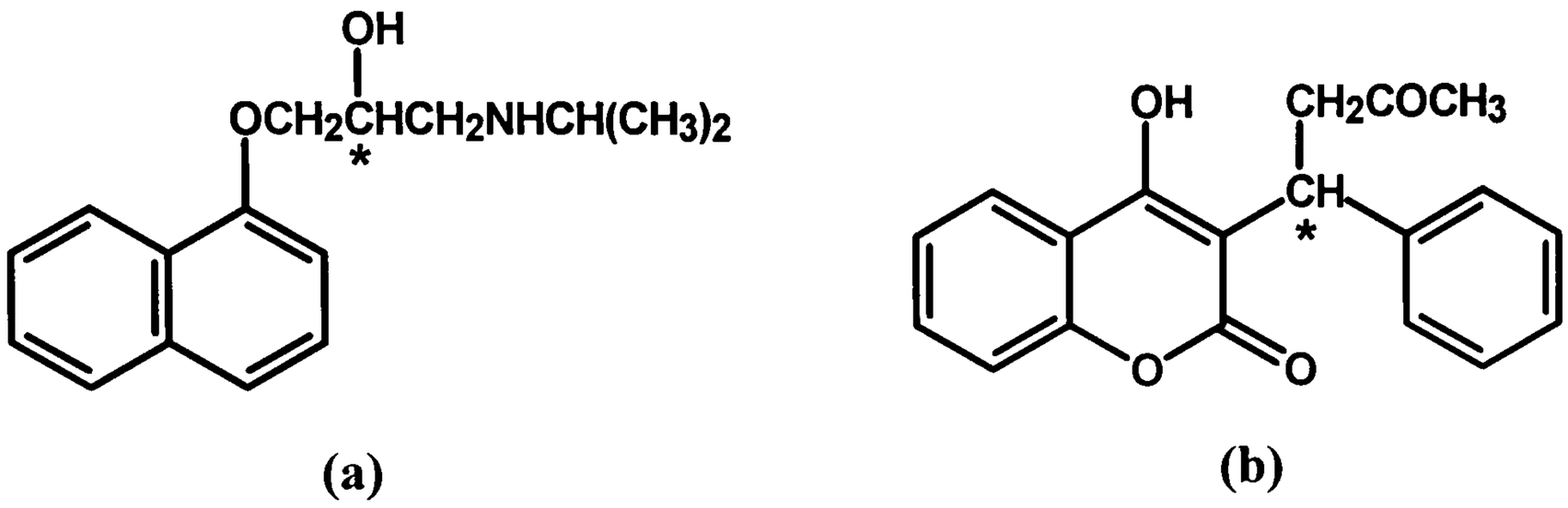


Figure 1.7: Structure of (a) propranolol and (b) warfarin.

Both enantiomers are marketed with different indications. Recognition some years ago of the different spectrum of activity of the enantiomers of propoxyphene (Figure 1.8) resulted in the development of two enantiomerically pure drugs: dextro-propoxyphene (Darvon), an analgesic and levopropoxyphene (Novrad), an antitussive. The trade names are, quite appropriately, mirror images of each other.

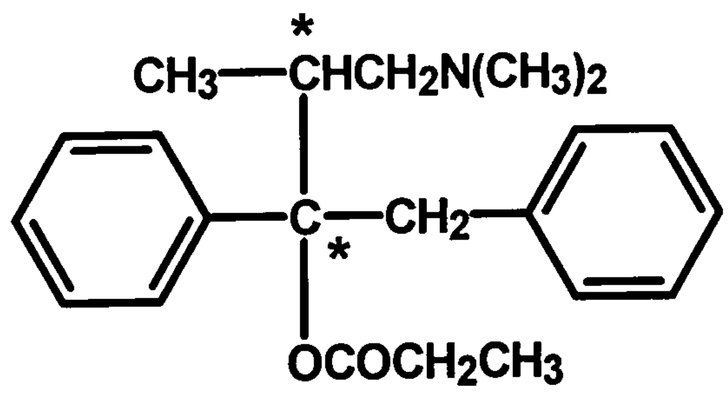


Figure 1.8: Structure of propoxyphene.

Toxicological activity resides predominantly in one enantiomer. There are several examples where one enantiomer contributes little to therapeutic efficacy but is mainly responsible for undesirable side-effects. Penicillamine (Figure 1.9) is used for the treatment of Wilson's disease, cystinuria and rheumatoid arthritis. Severe adverse

reactions such as nephritis are attributed to the L-enantiomer and, therefore, only the D-enantiomer is used (Williams, 1990). Ketamine (Figure 1.9) is a useful anaesthetic with analgesic properties and does not cause circulatory or respiration depression. However, (*R*)-ketamine is three to four fold less potent than its antipode and is responsible for the excitatory and hallucinogenic effects on emergence from anaesthesia (White *et al.*, 1980). Clearly, it would seem preferable to use (*S*)-ketamine, i.e. the more potent and less toxic enantiomer, rather than the racemic mixture and recently the single isomer product has been approved in Germany (Tucker, 2000). In a similar manner, bupivacaine (Figure 1.9), a long-acting amide local anaesthetic, has been re-marketed as the single *S*-enantiomer since it is less cardiotoxic than the antipode and the racemate (Tucker, 2000).

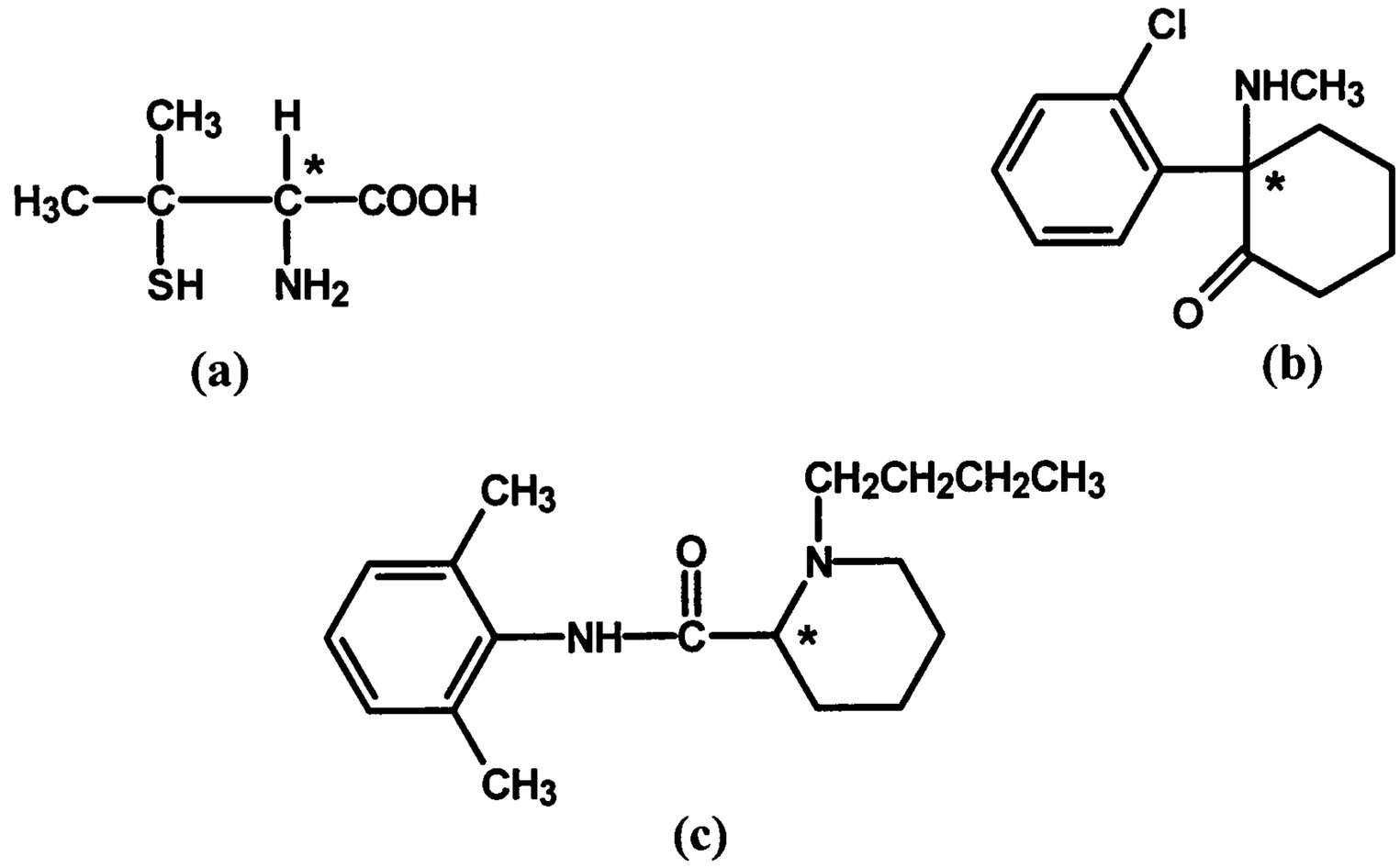


Figure 1.9: Structures of (a) penicillamine, (b) ketamine and (c) bupivacaine.

The enantiomers have opposite effects. Picenadol (Figure 1.10) is a phenylpiperidine derivative that has both opioid agonist and antagonist activity. (+)-(3*S*,4*R*)-Picenadol has a strong agonist activity at the μ receptor whilst the (-)-(3*R*,4*S*)-antipode has a weak antagonistic activity at the same receptor. The racemate therefore has moderate analgesic activity and acts as a partial agonist (Powell *et al.*, 1988).

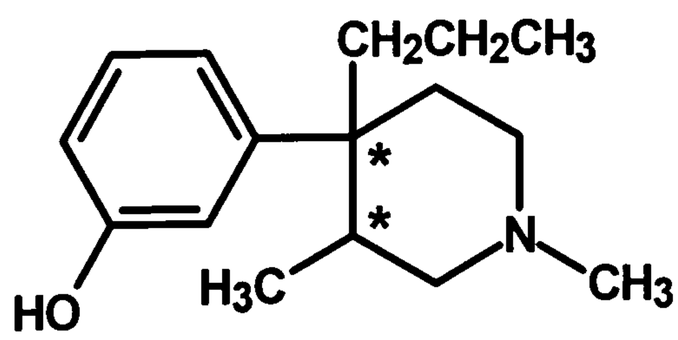


Figure 1.10: Structure of picenadol.

Toxicological activity of one enantiomer antagonised by other. Nebivolol (Figure 1.11) is a recently introduced β-blocker used for the treatment of hypertension. The (+)-isomer, which has the *S,R,R,R* configuration, is highly selective and has a long half-life. The (-)-isomer with the *R,S,S,S* configuration, has no β-blocking activity but is reported to moderate the negative inotropic effects of (+)-nebivolol (Van de Water *et al.*, 1988).

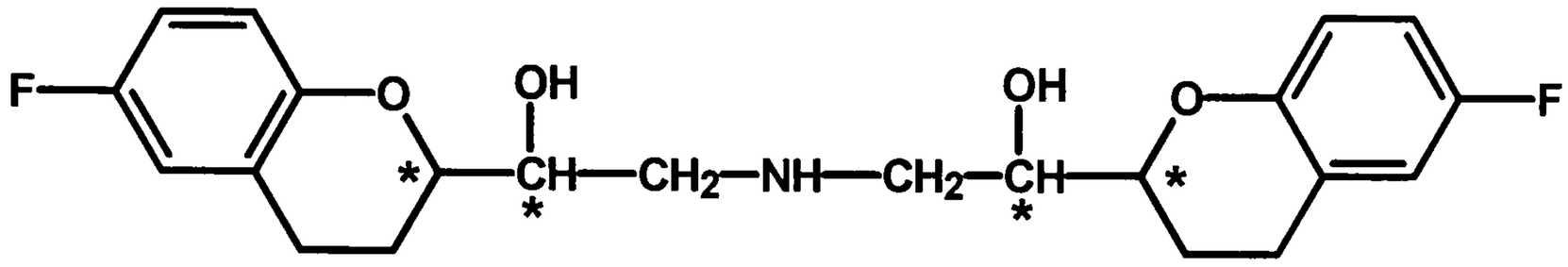


Figure 1.11: Structure of nebivolol.

1.3.2 Stereoselective pharmacokinetics

Differences between the pharmacological activity of enantiomers may be of a pharmacokinetic as well as of a pharmacodynamic origin (Caldwell *et al.*, 1988b; Jamali *et al.*, 1989; Tucker and Lennard, 1990; Levy and Boddy, 1991). Stereoselectivity may occur in virtually all phases of drug disposition, i.e. absorption, distribution, metabolism and excretion since many of these processes involve interaction of the drug enantiomers with chiral biological macromolecules. The magnitude of the differences between stereoisomers with respect to their pharmacokinetic parameters tends to be relatively modest, frequently one to three fold compared with those observed with respect to pharmacodynamic properties; however, they are of clinical significance.

i) Absorption

Drug absorption through the gastrointestinal tract, skin or the pulmonary system is for the majority of compounds a passive process which depends on factors such as lipophilicity, pKa and molecular size, and therefore the rate and extent of absorption do not differ between a pair of enantiomers as they have the same physicochemical properties. However, stereoselective differences may arise if the drug is a substrate for active or carrier-mediated transport systems. For example, the absorption of L-dopa and L-methotrexate is favoured over their respective antipodes (Wade *et al.*,1973; Hendel

and Brodthagen, 1984) as the L-isomers are absorbed by active transport mechanisms while the D-isomers are absorbed by passive diffusion.

Additional biochemical or pharmacological factors may influence the stereoselectivity of drug absorption. For example, the (-)-isomer of bupivacaine has a lower systemic absorption and longer-lasting local-anaesthetic activity than its antipode following intradermal injection, as consequence of its vasoconstrictor effect leading to a decrease in localised blood flow (Alps and Reynolds, 1978). In the case of terbutaline, the greater oral bioavailability of the more active (-)-isomer has been associated with stereoselective first-pass metabolism and may also be due to the (-)-isomer increasing intestinal membrane permeability (Brogstrom *et al.*, 1989).

However, stereoselective absorption of passively absorbed drugs can also occur if the enantiomers have difference dissolution profiles as a result of enantioselective intermolecular interactions with chiral excipients in the dosage form. For example, the release of propranolol from a formulation containing hydroxypropylmethylcellulose is stereoselective (Duddu *et al.*, 1993) and verapamil dissolution from modified release dosage forms can favour the *R*-enantiomer (Aubry and Wainer, 1993).

It is noteworthy, that so far little or no attention has been drawn to the relevance and stereoselectivity of the inverse transport (counter-transport) mechanisms of the gastrointestinal tract mediated by P-glycoprotein. Recent *in vivo* and *in vitro* data supports the hypothesis that (*S*)-talinolol is a slightly better substrate than the *R*-enantiomer for P-glycoprotein (Wetterich *et al.*, 1996). Hence, net absorption of (*S*)-talinolol into systemic circulation is reduced, and plasma concentrations are lower in comparison to its *R*-antipode (Wetterich *et al.*, 1996).

ii) Distribution

The extent of distribution of drugs is essentially governed by plasma protein binding, tissue protein binding and lipophilicity. As the latter is a physical property, the partition of a drug into various sites is not expected to be enantioselective, but the extent of binding of a pair of enantiomers however, may differ substantially.

The plasma protein binding of a drug is an important factor influencing both pharmacodynamics and pharmacokinetics since only the drug in the unbound-form is responsible for pharmacological action and available for distribution to tissues and clearance. For highly protein bound drugs, minor enantiomeric differences in binding leads to much larger differences in the enantiomeric composition of the unbound

fraction. The majority of drugs bind in a reversible manner to plasma proteins, notably to human serum albumin (HSA) and /or α_1-acid glycoprotein (AGP). Acidic drugs bind preferentially to HSA, with binding at site II (benzodiazepine site) on the protein generally displaying greater enantiomeric differences than at site I (warfarin site) (Fehske *et al.*, 1981). Example of stereoselective plasma protein binding for acidic drugs include ibuprofen, indacrinone, phenprocoumon, warfarin, mephobarbitone and pentobarbitone (Hutt, 1998). Basic drugs predominately bind to AGP and stereoselective binding has been reported for numerous drugs including chloroquine, disopyramide, methadone, mexiletine and verapamil (Hutt, 1998). Stereoselectivity in binding may vary for different proteins, e.g. the protein binding of propranolol to AGP is stereoselective for the *S*-enantiomer, whereas binding to HSA favours the *R*-antipode (Hutt, 1998). In whole plasma the binding to AGP is dominant such that the free fraction of (*R*)-propranolol is greater than that of (*S*)-propranolol (Hutt, 1998).

Competition for plasma protein binding sites is a common cause of pharmacokinetic complications. For example, enantiomer-enantiomer interactions in the concentration-dependent plasma protein binding of disopyramide following administration of the racemate, exposed enantiomeric differences in disposition that were not evident following separate administration of the enantiomers (Giacomini *et al.*, 1986). The co-administration of another drug can also influence the protein binding of a chiral drug, e.g. phenylbutazone administration causes a greater displacement of (*R*)- compared to (*S*)-warfarin from plasma protein binding sites but concomitantly inhibited oxidative metabolism of the *S*-enantiomer to a larger extent. The cumulative effect is an increased total clearance of (*R*)-warfarin; but more significantly, a reduced clearance of active (*S*)-warfarin leading to enhanced pharmacological activity (Hutt, 1998).

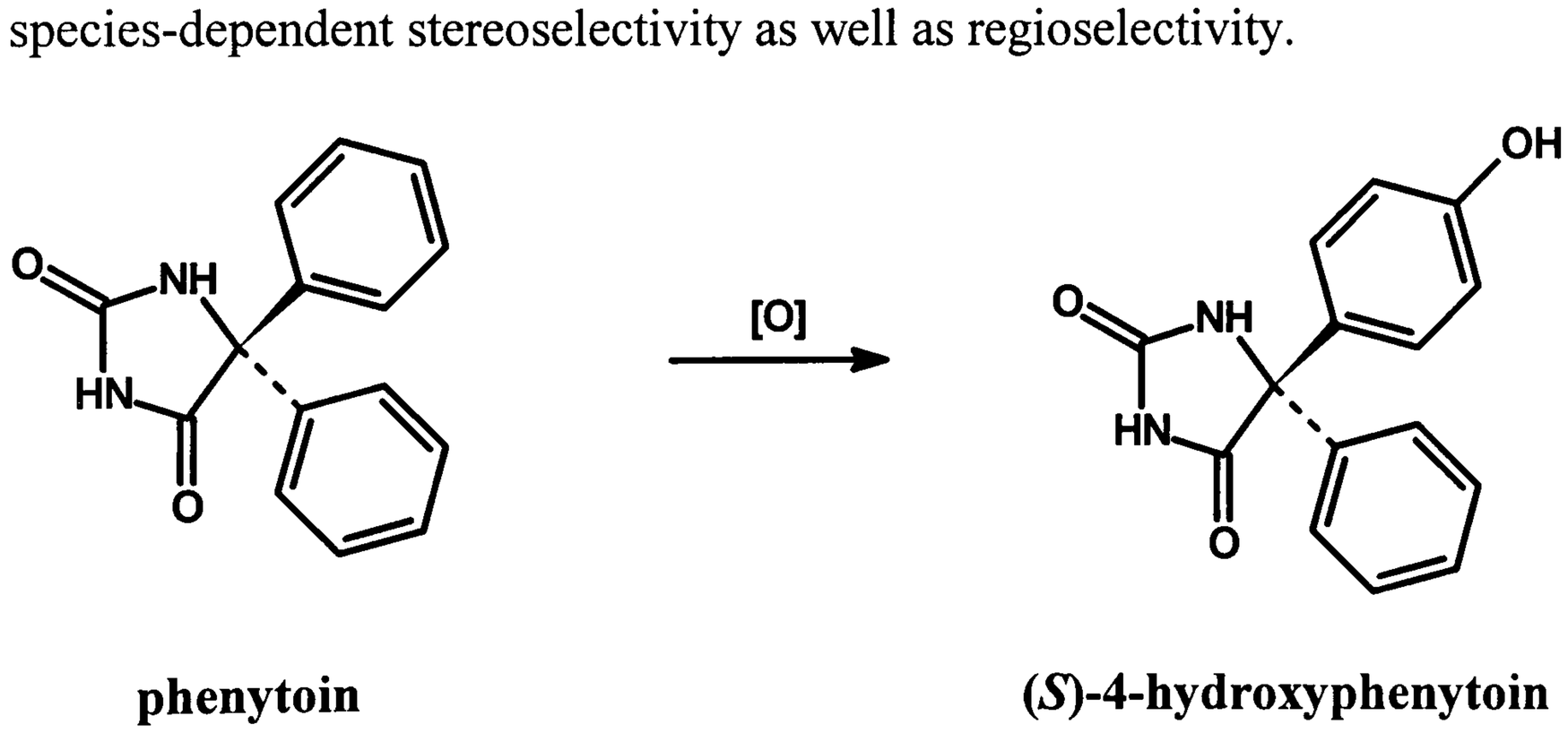
Enantioselective tissue binding, which is in part a consequence of enantioselective plasma protein binding, has been reported. For example, the transport of ibuprofen into both synovial and blister fluids is preferential for the *S*-enantiomer owing to the higher free fraction of this enantiomer in plasma (Seideman *et al.*, 1994). In addition the affinity of stereoisomers for binding sites in specific tissues may also differ and contribute to stereoselective tissue binding, e.g. (*S*)-leucovorin accumulates in tumour cells *in vitro* to a greater degree than the *R*-antipode (Mader *et al.*, 1994). The uptake of ibuprofen into lipids is stereoselective in favour of the *R*-enantiomer, but this is as a

result of stereospecific formation of the acyl-CoA thioester followed by incorporation as hybrid triglycerides (see section 1.4.2).

iii) Metabolism

Metabolism tends to exhibit the greatest degree of stereoselectivity of all the processes contributing to drug disposition, with both phase I and II reactions potentially capable of discriminating between enantiomers (Caldwell *et al.*, 1988b). The chirality of both the parent drug and metabolite have to be considered when stereoselective metabolism is discussed, five scenarios are possible:

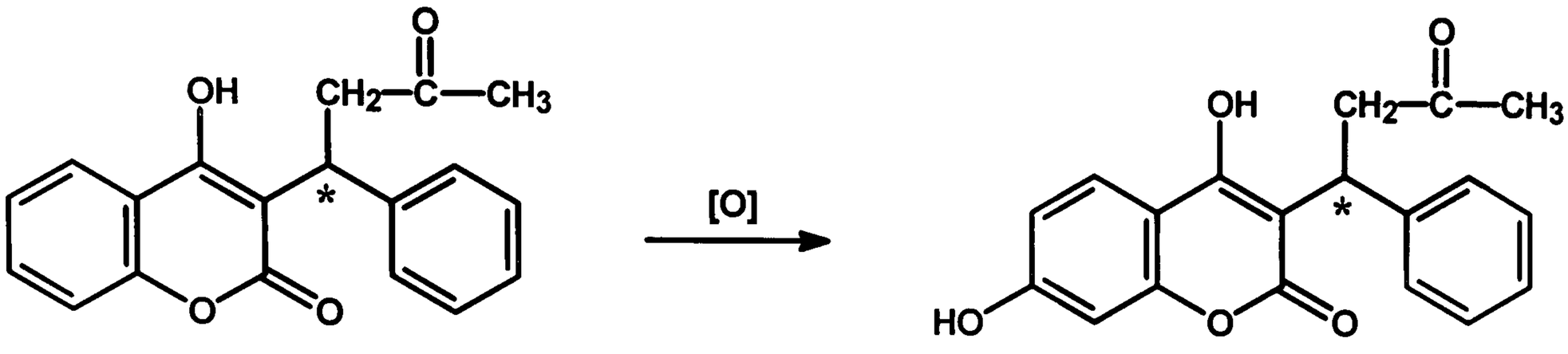
Prochiral to chiral transformations, where a prochiral molecule is metabolised to preferentially form one of two possible enantiomers. An example of this type of reaction is the *p*-oxidation of the antiepileptic drug phenytoin, which displays significant product selectivity in favour of the formation of (*S*)-4-hydroxyphenytoin in man (Figure 1.12) (Poupaert *et al.*, 1975). Whereas in the dog, phenytoin undergoes oxidation to yield the *R*-enantiomer of the *m*-hydroxylated product (Maguire *et al.*, 1978) and thus the reaction shows species-dependent stereoselectivity as well as regioselectivity.



phenytoin **(*S*)-4-hydroxyphenytoin**

Figure 1.12: Prochiral to chiral transformation, oxidation of phenytoin.

Chiral to chiral transformations, in which the two enantiomers of a drug are differentially metabolised at a site remote from the chiral centre. With warfarin, aromatic oxidation to form the 7-hydroxy metabolite is highly selective for the more active *S*-enantiomer in man (Figure 1.13) (Lewis *et al.*, 1974).



warfarin **7-hydroxywarfarin**

Figure 1.13: Chiral to chiral transformation, aromatic oxidation at the 7-position of warfarin.

Chiral to achiral transformations, where the chiral substrate undergoes metabolism leading to loss of asymmetry. Although, not frequently encountered, it is nevertheless important especially if substrate stereoselectivity is observed. For example nivadipine, which has a dihydropyridine structure, undergoes cytochrome P450-mediated oxidation to yield the corresponding achiral pyridine analogue (Figure 1.14); the reaction is stereoselective for the (-)-enantiomer of the drug in man (Hutt, 1998).

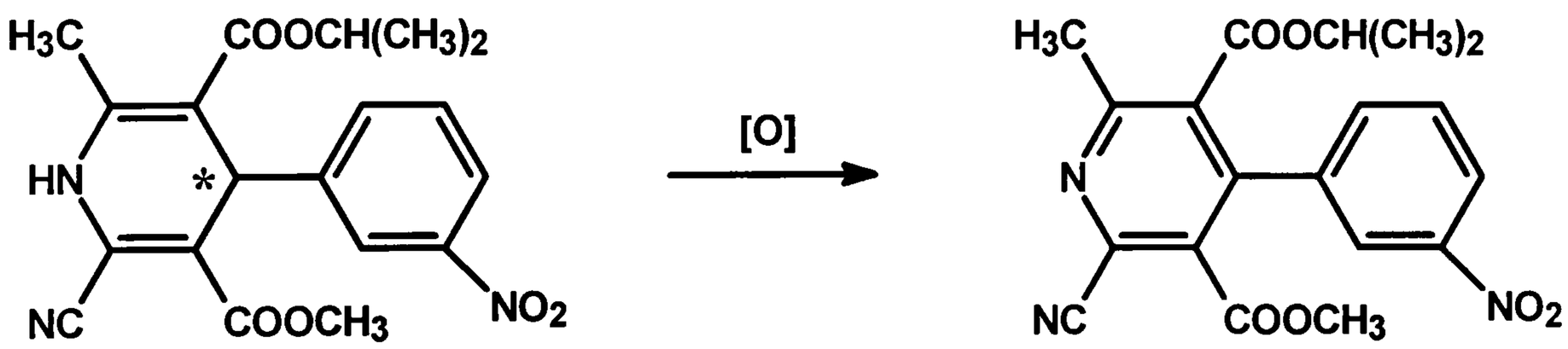


Figure 1.14: Chiral to achiral transformation, aromatization of nilvadipine.

Chiral to diastereoisomer transformations, where either phase I or phase II metabolism introduces a second chiral centre into the molecule. An example of the first type is the keto-reduction of warfarin to yield diastereomeric alcohols (Figure 1.15), this reaction shows substrate selectivity for (*R*)-warfarin and favours the *S*-configuration at the new chiral centre (Hutt, 1998). Phase II metabolic reaction of a chiral substrate with a chiral conjugating agent, such as D-glucuronic acid, glutamine and glutathione, will inevitably produce diastereomeric products. In man, oxazepam conjugation with D-glucuronic acid shows preference for the *S*-enantiomer of the drug (Figure 1.15) (Sisenwine *et al.*, 1982).

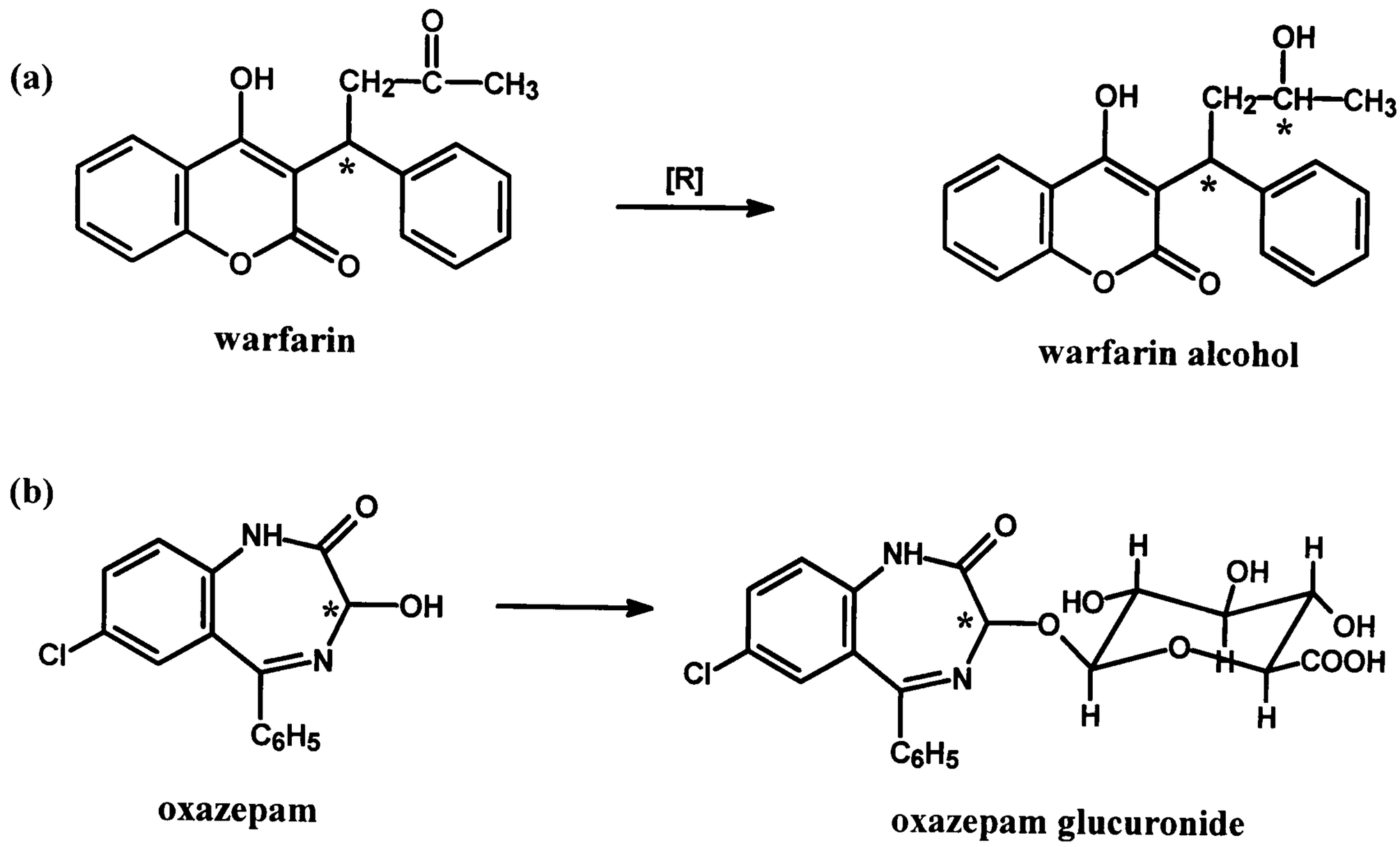


Figure 1.15: Chiral to diastereoisomer transformations, (a) keto-reduction of warfarin and (b) glucuronidation of oxazepam.

Chiral inversion, where one enantiomer of the substrate is biochemically converted into its antipode with no other alterations in structure. Such a transformation is relatively rare since it necessitates the breaking of a bond between the chiral centre and a group directly attached before subsequent reformation of the bond in the alternative configuration. The reaction was first observed with the 2-arylpropionic acid NSAIDs, with the inactive *R*-enantiomers undergoing selective inversion to their active *S*-antipodes in many animal species including man (Hutt and Caldwell, 1983). The mechanism is thought to proceed via formation of (*R*)-profenyl-CoA-thioesters that subsequently epimerize and hydrolyse to liberate both enantiomers of the drug. The *S*-enantiomer is unable to undergo thio-esterification and so the inversion process is unidirectional (Nakamura *et al.*, 1981). The chiral inversion of 2-arylpropionic acid drugs will be examined in detail in section 1.4.2.

iv) Renal excretion

Renal excretion is the composite effect of glomerular filtration, active secretion and passive and active reabsorption. Apparent stereoselectivity in renal clearance may arise as a consequence of stereoselective protein binding leading to enantiomeric differences

in glomerular filtration and/or passive reabsorption. Active renal tubular secretion has been associated with stereoselective clearance of some basic drugs including terbutaline, disopyramide, chloroquine and pindolol (Hutt, 1998). It is also possible that enantiomeric differences may originate from stereoselectivity in active reabsorption or renal metabolism. However, stereoselective renal elimination tends to have little impact on the disposition of the enantiomers unless this is a major clearance pathway of the drug.

As a result of stereoselectivity in the various processes of drug disposition outlined above, the pharmacokinetic profile of a pair of enantiomers may differ markedly. Thus an estimation of pharmacokinetic parameters and concentration-effect relationships based on total drug concentrations are of limited value and potentially misleading, and have been described as "sophisticated nonsense" (Ariens, 1984). Furthermore, whilst a considerable number of enantioselective pharmacokinetics studies have been performed, little is known about the influence of various patient factors, e.g. age, sex, disease state and pharmacogenetics, on stereochemical aspects of drug disposition.

1.3.3 Current implications of drug stereochemistry

From the above it is apparent that individual enantiomers may differ markedly in their pharmacodynamic and pharmacokinetic profiles and thus their presence in a racemic mixture can not be viewed simply as a 50:50 mixture of an active drug and an inert component. The use of racemates is regarded by some as "polypharmacy", with the proportions in the mixture being dictated by chemical rather than therapeutic considerations; such agents are also regarded as compounds containing 50% impurity (Ariens, 1984; 1986). To date there is a large body of information regarding the stereoselective differences in the action and disposition of chiral drugs which supports the view that the use of racemic mixtures may have important pharmacological and toxicological implications. The strength of evidence has prompted regulatory agencies to promote the use of single isomer preparations for new chemical entities unless it can be proven that the racemate gives therapeutic benefits over the single enantiomer or that

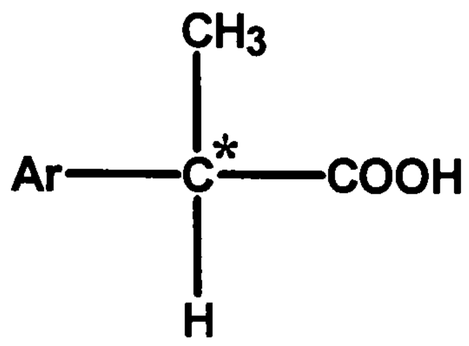
the preparation of a single isomer is not technically feasible on an industrial scale (Langaniere, 1997; Williams *et al.*, 1998).

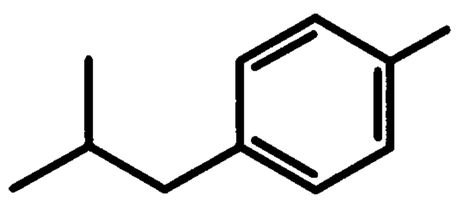
Furthermore, a re-evaluation of drugs marketed as racemates has led to a few successful "chiral switches", i.e. commercialisation of a single enantiomer product from a previously marketed racemate (Tucker, 2000). Situations where a racemate-to-enantiomer switch may be appropriate include: drugs where there are problems associated with the racemate or it would be therapeutically advantageous to use the single isomer, pharmacological activity resides in one isomer, the mode of action established for the racemate is valid for the single isomer and synthesis or resolution of the desired isomer is feasible at the appropriate scale (Caldwell, 1999). In terms of drug group, the most significant switches have been observed for the 2-arylpropionic acid NSAIDs with the *S*-enantiomer of ibuprofen available in Austria and Switzerland, and (*S*)-ketoprofen marketed in Spain and recently introduced in the United Kingdom. However as yet, the majority of the drugs in this group continue to be marketed and used as racemic preparations. Therefore, an examination of stereochemical aspects of the pharmacodynamic and pharmacokinetic properties of such drugs is essential for their rational therapeutic re-assessment, especially since they display stereoselectivity in action, protein binding, chiral inversion and metabolism. Moreover, 2-arylpropionic acid drugs are most widely used in the elderly population where there are likely to be age-related alterations in drug handling, and co-morbid disease conditions and multiple drug therapies are likely to be more prominent.

1.4. The 2-arylpropionic acid group of anti-inflammatory drugs

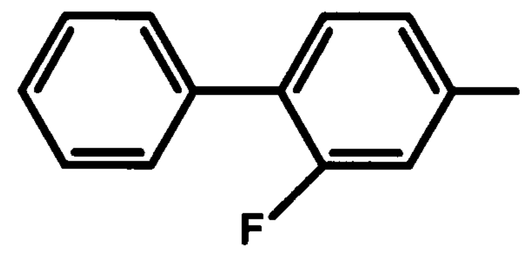
Non-steroidal anti-inflammatory drugs (NSAIDs) are extensively prescribed for alleviating pain and inflammation associated with a wide range of joint and muscoskeletal disorders. As a group, the NSAIDs encompass a variety of structural classes including salicylates, pyrazoles, oxicams, fenamates, arylacetic acids and arylpropionic acids. Of these subcategories, the 2-arylpropionic acids are the most frequently used agents and have been the backbone of anti-inflammatory drug treatment since ibuprofen became commercially available in 1969 in the UK (Williams *et al.*,

1993). Subsequently, numerous analogues have been developed and the structures of some are shown in Figure 1.16.

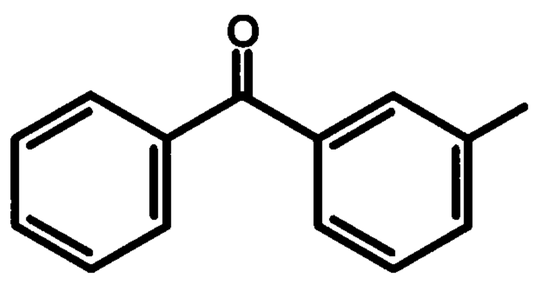




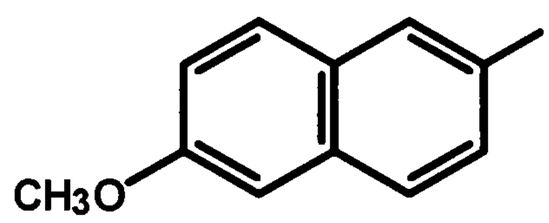
Ibuprofen



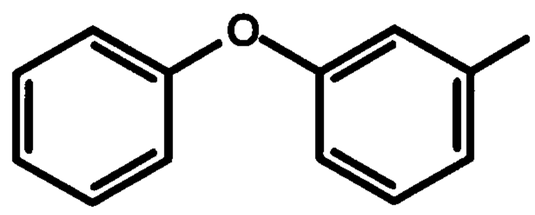
Flurbiprofen



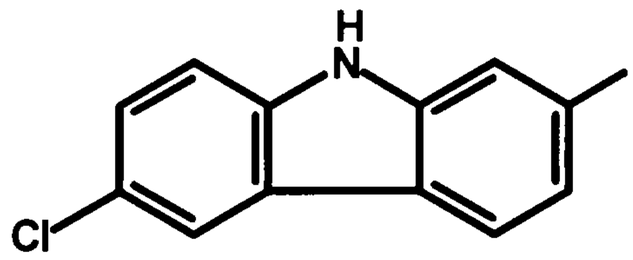
Ketoprofen



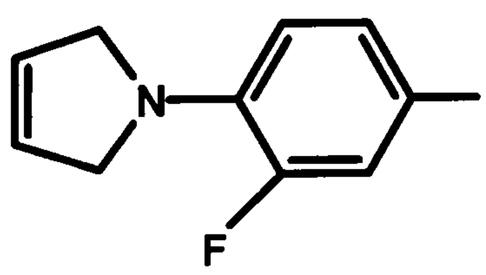
Naproxen



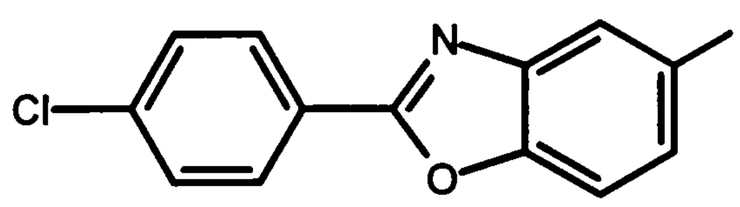
Fenoprofen



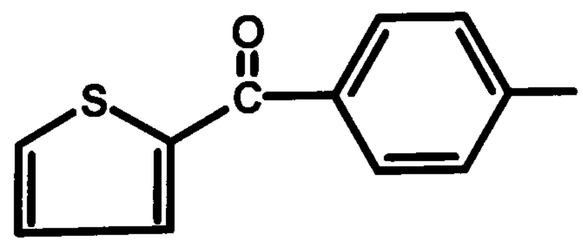
Carprofen



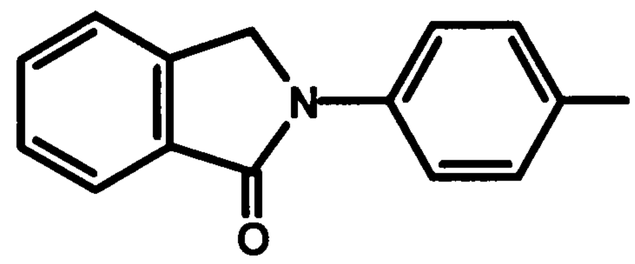
Pirprofen



Benoxaprofen



Suprofen



Indoprofen

Figure 1.16: Chemical structures of some 2-arylpropionic acid analogues.

Drugs belonging to the 2-arylpropionic acid class have certain common structural components essential for anti-inflammatory activity. These include a carboxylic acid functionality and a substituted aromatic group attached to the α-carbon atom, and arrangement of the four different substituents around the α-carbon must be in the *S*-configuration to afford activity. Indeed, it was the early recognition that activity resided in the one enantiomer which prompted the development of the successful enantiomerically pure drug, (*S*)-naproxen, in the early 1970s. The majority of these agents are still used in their racemic form; however, as outlined above, the current stereochemical consciousness has led to chiral switches of ibuprofen and ketoprofen in some countries.

1.4.1 Pharmacodynamics

Cyclooxygenase inhibition

It has been generally accepted that the 2-arylpropionic acids and other NSAIDs exert most of their pharmacological and toxicological effects by inhibiting the binding of arachidonic acid to cyclooxygenase (COX), thus preventing the formation of pro-inflammatory prostaglandins such as prostaglandin E_2 (PGE_2; Figure 1.17) (Vane, 1971; Fereira and Vane, 1979). The *in vitro* COX inhibitory activity of the 2-arylpropionic acids resides principally in the enantiomer of the *S*-configuration, as reflected by high eudismic ratios. Comparison of the relative potencies of the enantiomers in *in vivo* test systems however show that the large eudismic ratios observed *in vitro* are diminished such that in some cases, e.g. ibuprofen and fenoprofen, both enantiomers display approximately equal activity (Table 1.1). These disparities arise due to *in vivo* chiral inversion, which involves the metabolism of the relatively inactive *R*-enantiomer of the drug to the pharmacologically active antipode. In fact, a large difference between *in vivo* and *in vitro* eudismic ratios is a good indicator that metabolic chiral inversion has occurred (Adams *et al.*, 1976).

Recently, COX has been shown to be encoded by two genes giving rise to two distinct forms of this enzyme, namely COX-1 and COX-2 (Vane *et al.*, 1998; Wu, 1998). COX-1 is expressed in most tissues under normal physiological conditions and is associated with cellular homeostatic, i.e. normal "housekeeping", functions in for

example the gastrointestinal tract and kidneys (Vane *et al.*, 1998). In contrast, COX-2 is chiefly produced in inflammatory cells in response to tissue trauma and inflammation and is also expressed constitutively in brain neurons and regions of the kidney (Vane *et al.*, 1998). Therefore, the prostaglandins generated following stimulation of COX-2 activity are most likely to be responsible for pain and tissue damage (Dray and Bevan, 1993).

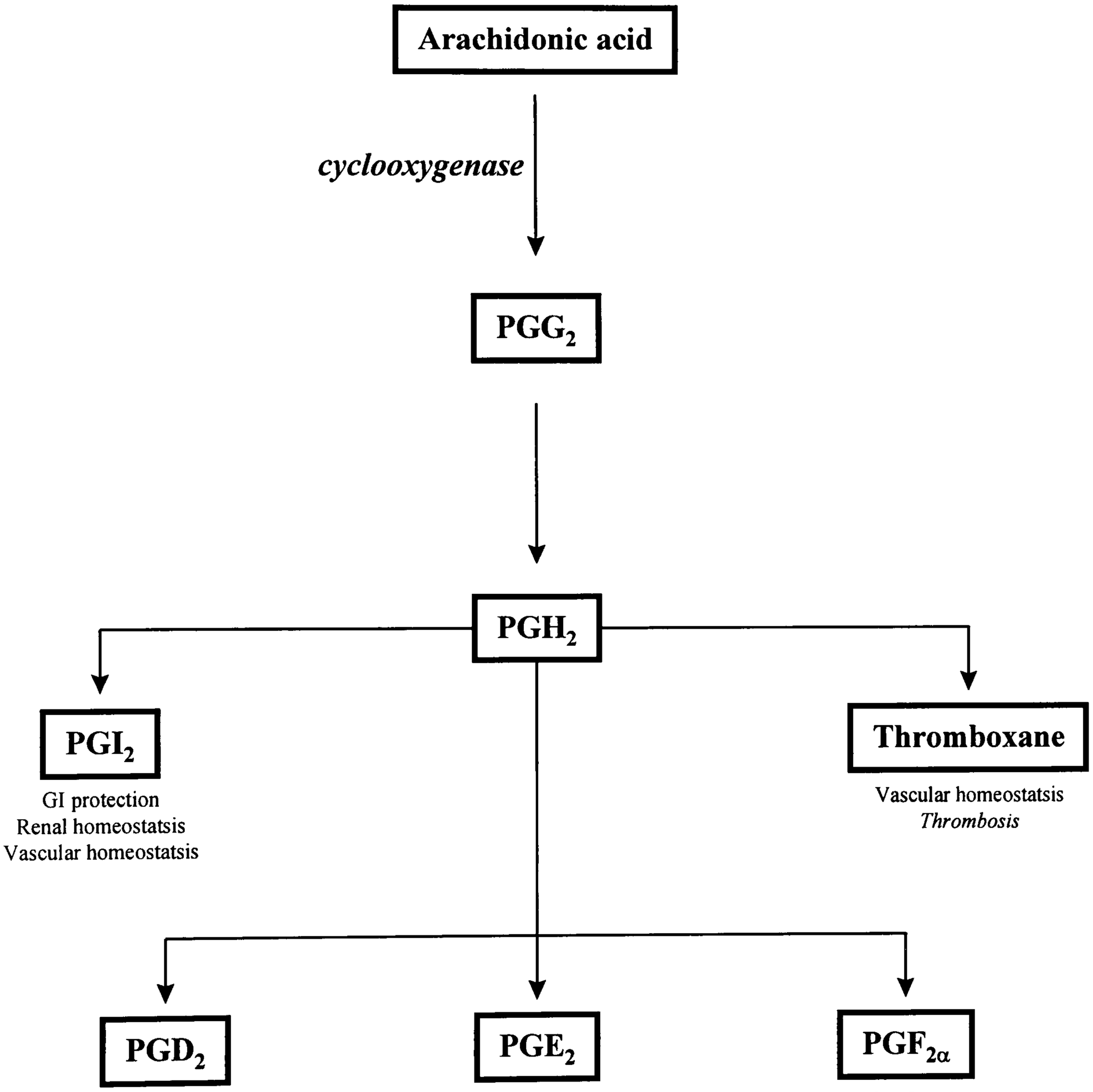


Figure 1.17: The arachidonic acid cascade (adapted from Vane *et al.*, 1998).

Table 1.1: Relative activity of the enantiomers of 2-arylpropionic acid NSAIDs in *in vitro* and *in vivo* test systems (adapted from Hutt, 1998).

Drug	Ratio *S/R*		Test System
	in vitro	*in vivo*	
Carprofen	> 24		prostaglandin synthesis inhibition
		14	acute adjuvant arthritis
Fenoprofen	35		platelet aggregation inhibition
		1	carrageenin paw oedema
Flurbiprofen	200		platelet aggregation inhibition
		2 to 16	guinea-pig anaphylaxis
Ibuprofen	160		prostaglandin synthesis inhibition
		1.1	ultraviolet induced erythema
Naproxen	130		prostaglandin synthesis inhibition
		28	carrageenin paw oedema

Following the identification of COX-1 and COX-2, the degree to which NSAIDs differentially inhibit the two isoforms has been evaluated in a wide variety of *in vitro* test systems. However, a major drawback has been the variability in results between different assay methods in terms of both the degree of COX-inhibition and the COX-2 to COX-1 selectivity. Recently, Warner *et al.* (1999) developed an improved COX selectivity assay method which took into consideration a number of important criteria for optimum reliability, these were: (1), the use of both isoforms from a common species (preferably human); (2), common incubation times of drugs with both COX systems and (3), equivalent levels of substrate being available to both isoforms. The COX-2 to COX-1 selectivity, in terms of the ratio of IC_{80} values, determined for aspirin and various 2-arylpropionic acids using this novel method are presented in Figure 1.18. The degree of COX selectivity was quite varied between the different 2-arylpropionic acids, although all showed preference towards inhibiting COX-1.

It has been suggested that selective COX-2 inhibition, at the expense of COX-1 blockade would be a logical therapeutic goal, thereby limiting the well recognised gastrointestinal adverse effects of 2-arylpropionic acids and other NSAIDs. Indeed, numerous *in vitro* investigations of the COX-selectivity of NSAIDs have shown that agents with less inhibitory activity for COX-1 are less likely to cause gastrointestinal toxicity (Hayller and Bjarnason, 1995; Kawai *et al.*, 1998; Warner *et al.*, 1999). Such *in*

vitro observations should be related with caution to the clinical situation, in view of the multifactorial nature of the pathophysiology of NSAID-induced adverse effects and also the fact that *in vivo*, other factors such as enantioselective disposition have to be considered. The situation could be further complicated by recent *in vitro* evidence which suggests that the coenzyme-A thioester of (*R*)-ibuprofen, which is an intermediate of the chiral inversion process, also possesses significant COX-2 inhibitory activity (Neupert *et al.*, 1997).

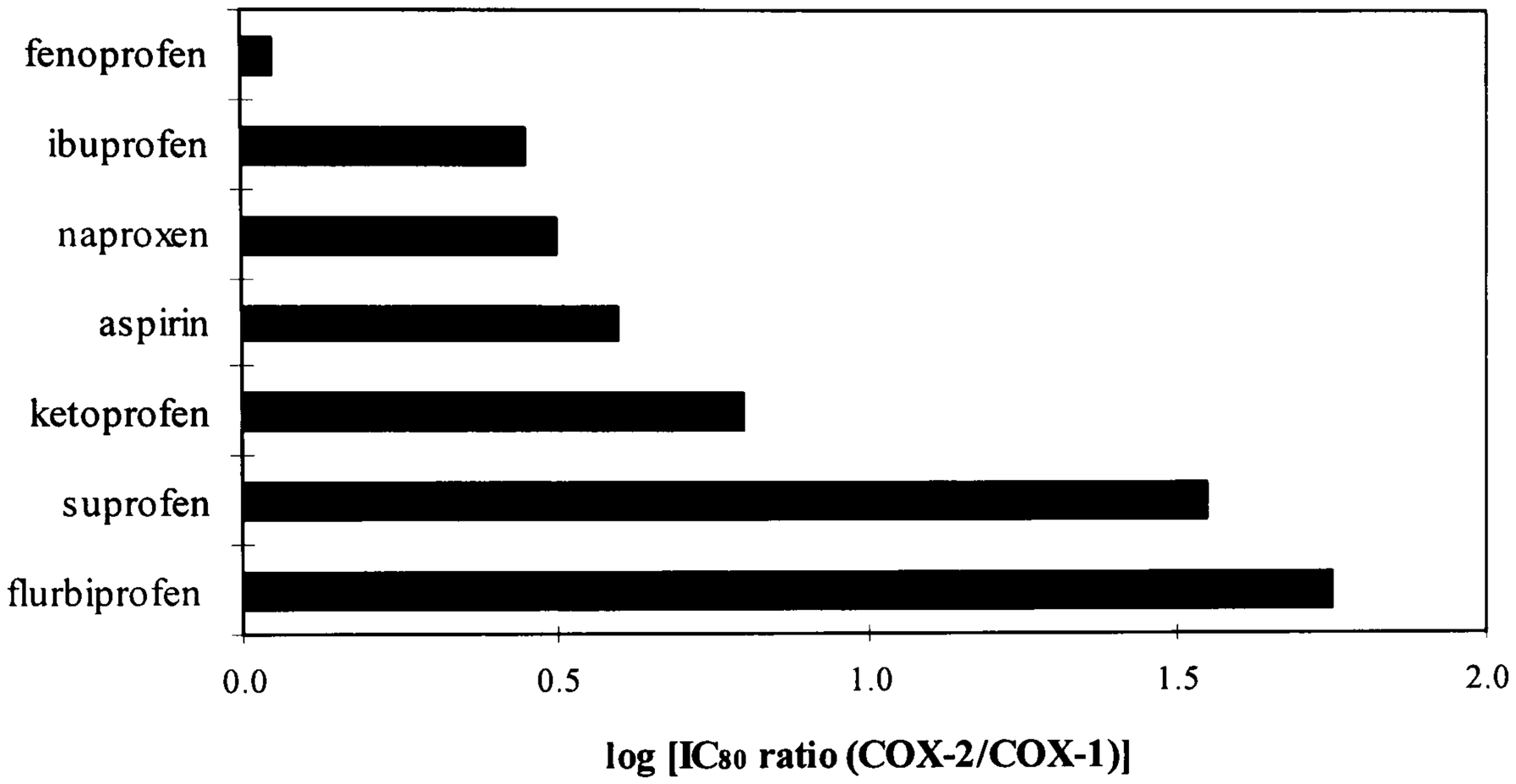


Figure 1.18: Relative activities against COX-1 versus COX-2 for aspirin and some 2-arylpropionic acids (adapted from Warner *et al.*, 1999).

Nevertheless, recent randomised double-blind trials of two COX-2 specific inhibitors, i.e. celecoxib (Simon *et al.*, 1999) and rofecoxib (Langman *et al.*, 1999), showed that treatment with these drugs was associated with a lower incidence of gastrointestinal complications than treatment with non-specific NSAIDs. However, only widespread use will confirm or refute their utility over traditional NSAIDs and the premise that selective COX-2 inhibitors will not affect the gastrointestinal tract has already been challenged. In three experimental models, COX-2 selective drugs were observed to inhibit angiogenesis, i.e. formation of new capillary blood vessels, which is required for ulcer healing (Jones *et al.*, 1999). To further support the notion, it was demonstrated that L-745,337, a selective COX-2 inhibitor, delayed the healing of gastric ulcers partly through the inhibition of angiogenesis (Jones *et al.*, 1999).

Non-cyclooxygenase activity

Although the anti-inflammatory action of 2-arylpropionic acids and other NSAIDs appears to result from inhibition of COX, several recent reports suggest that non-COX mechanisms may also be important in the analgesic action of these drugs (Brune *et al.*, 1991, 1992; Geisslinger *et al.*, 1994a; Lötsch *et al.*, 1995; Neugebauer *et al.*, 1995; Geisslinger and Schaible, 1996, Buritova and Besson, 1998). These reports focus on the fact that in different models of animal and human nociception, the pure *R*-enantiomer of flurbiprofen, an ineffective COX inhibitor, possesses substantial analgesic activity. These findings are pivotal since (*R*)-flurbiprofen undergoes little if any chiral inversion when given to rats or humans. Further support for the pharmacological benefits of the poorly inverted *R*-enantiomers of 2-arylpropionic acids, is provided by the effectiveness of (*R*)-ketoprofen as an analgesic in the treatment of post-operative dental pain (Cooper *et al.*, 1998) and the suggestion that (*R*)-ketoprofen is a more effective analgesic than its antipode (Ghezzi *et al.*, 1998). Highly lipid soluble drugs such as flurbiprofen and ketoprofen are likely to have the ability to cross the blood-brain barrier and central mechanisms of analgesic activity have been proposed. These include the ability to interfere with central opioid mechanisms, inhibition of serotonin activity and inhibitory activity on excitatory amino acids or NMDA receptors (Cashman, 1996).

In addition to inhibiting the synthesis of prostaglandins, NSAIDs in general exert a range of COX-independent biological actions, some of which may be important contributors to the pharmacological profile of the drug. Such activities include the ability to reduce *in vitro* sulphated glycosaminoglycan synthesis in articular cartilage, the ability to suppress neutrophil aggregation and degranulation, inhibition of inflammatory oedema by action on polymorphonuclear leukocytes and inhibition of mitochondrial β-oxidation (Evans, 1996). It is thought that many of these prostaglandin independent effects arise from interference with normal cell signalling processes due to their ability to partition into cellular phospholipid bilayer and disrupt G protein-dependent events. Membrane partitioning is unlikely to be enantioselective and so these COX-independent actions are invariably non-discriminatory between drug enantiomers. This is supported by a report of equipotent *in vitro* inhibition of human polymorphonuclear cell function by (*R*)-, (*S*)- and (*R,S*)-ibuprofen (Villaneuva *et al.*, 1993).

1.4.2 Pharmacokinetics

Absorption

Among the general pharmacokinetic features of the 2-arylpropionic acids is the rapid and efficient oral absorption of conventional doses. For example, the oral bioavailability of (*R,S*)-ibuprofen and (*R,S*)-flurbiprofen have been estimated to be approximately 0.95 and 0.94 respectively, indicating virtually complete absorption from the gastrointestinal tract and insignificant first-pass metabolism (Hall *et al.*, 1993; Szpunar *et al.*, 1987). As absorption of these drugs is governed by passive diffusion, enantiomeric differences in the systemic availability of 2-arylpropionic acids is unlikely (Evans, 1992).

Distribution

Once in the systemic circulation, the 2-arylpropionic acids are extensively bound (> 99%) to plasma proteins, predominately to albumin (Lin *et al.*, 1987). For ibuprofen, indoprofen and 2-phenylpropionic acid, plasma protein binding is greater for the *R*-enantiomer (Lapicque *et al.*, 1993). Whilst conflicting results have been reported for flurbiprofen (Knadler *et al.*, 1989; Blouin *et al.*, 1993) and ketoprofen (Hayball *et al.*, 1991; Dubois *et al.*, 1993a). By virtue of their extensive binding to plasma proteins, the 2-arylpropionic acids have rather small volumes of distribution, usually between 7 to 14 L in man (Evans, 1992). The high plasma protein binding also restricts the rate of movement of the 2-arylpropionic acids into the synovial fluid, and presumably, the central nervous system. Indeed the slow passage of ibuprofen enantiomers in and out of the joint may be responsible, in part, for the dampened and sustained concentration profiles in synovial fluid when compared to the plasma profiles (Day *et al.*, 1988). As trans-synovial transport appears to be a diffusion-controlled process, the higher concentrations of the *S*-enantiomer of ibuprofen in the synovium is likely to be partly reflective of the higher unbound concentrations of this enantiomer in circulation (Day *et al.*, 1988). In patients with rheumatoid arthritis who took (*R,S*)-flurbiprofen every twelve hours, the enantiomeric ratio (*S/R*) of the drug within synovial fluid increased from 1.8, 3 hours after the last dose to 1.47 at the end of the dosage interval, reflecting similar changes in the enantiomeric composition of the drug in plasma (Young *et al.*, 1991). As would be expected, the flurbiprofen enantiomers also had longer half-lives for elimination from synovial fluid than plasma (Young *et al.*, 1991).

Chiral inversion

The 2-arylpropionic acids have the ability to undergo unidirectional metabolic chiral inversion from the less active *R*-enantiomer to their more active *S*-antipodes (Hutt and Caldwell, 1983). It appears that this reaction is a general feature of the disposition of the 2-arylpropionic acids, although there can be substantial differences in both the rate and extent of inversion, which appears to vary with both the substrate and the species (Hutt and Caldwell, 1983, 1984; Caldwell *et al.* 1988a, 1988b). Thus, in man the degree to which inversion occurs may be negligible, as is the case with flurbiprofen (Jamali *et al.*, 1988); intermediate, for example ibuprofen (Lee *et al.*, 1984) or essentially complete, as with fenoprofen (Rubin *et al.*, 1985). As a result of a number of *in vitro* and *in vivo* investigations, the biochemical mechanism of the chiral inversion process has now become reasonably well defined and involves three main steps (Figure 1.19).

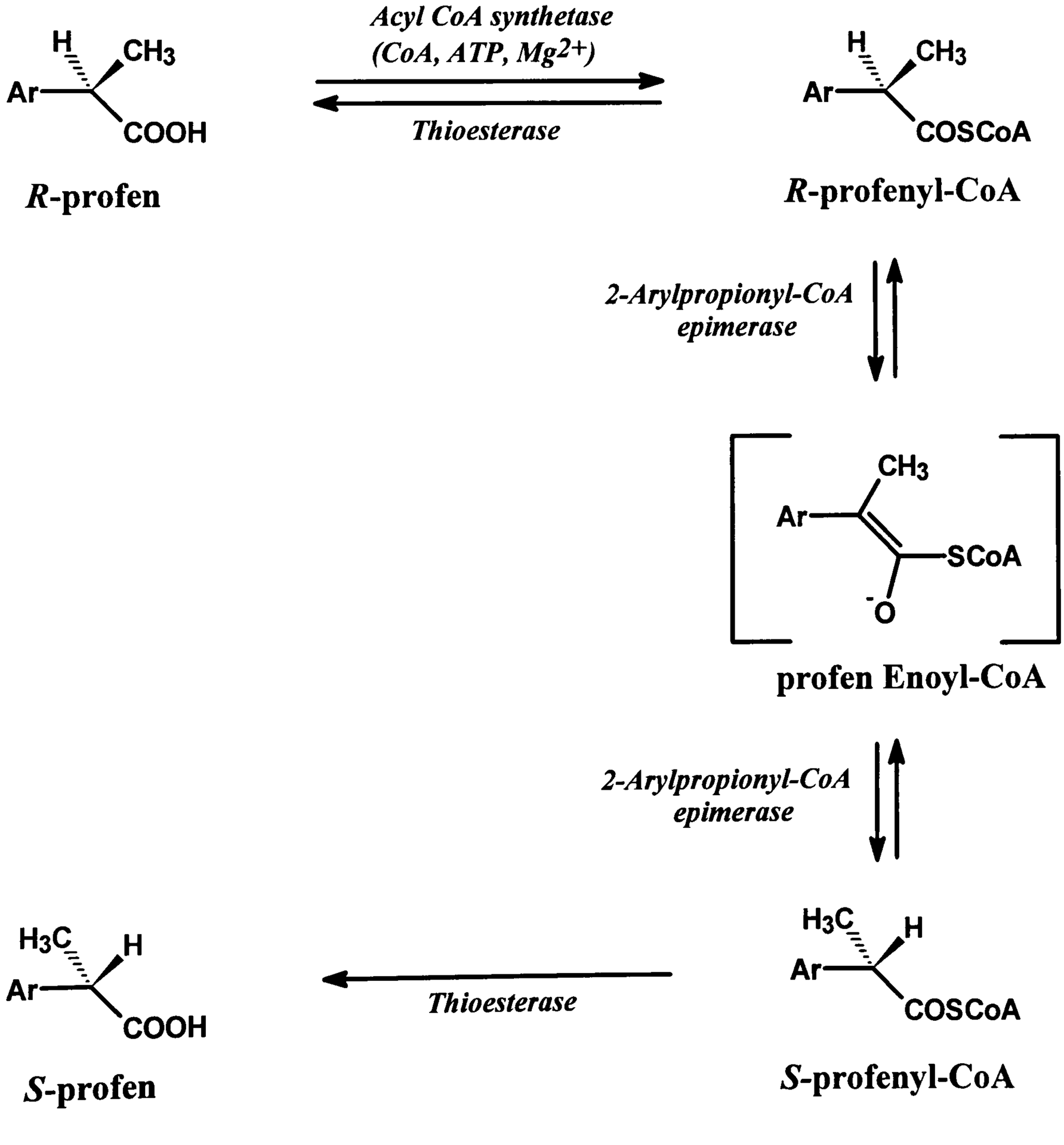


Figure 1.19: Mechanism of the metabolic chiral inversion of 2-arylpropionic acid derivatives.

The initial thioesterification of the *R*-enantiomer of the 2-arylpropionic acid with coenzyme A (CoA) via an adenylate intermediate is catalysed by a microsomal and mitochondrial enzyme, which has been characterised as long-chain acyl-CoA synthetase (Tracy *et al.*, 1993; Menzel *et al.*, 1994; Brugger *et al.*, 1996). The undirectional formation of the respective adenylates and/or the CoA thioester by the *R*-enantiomers is the stereoselective step of the inversion. Subsequently, the resulting *R*-profenyl-CoA thioester undergoes epimerisation of the chiral centre in the profenyl moiety, which is thought to proceed via an enolate intermediate to yield the corresponding *S*-profenyl-CoA thioester (Chen *et al.*, 1991; Tracy and Hall, 1992). The epimerase enzyme has been isolated from the cytosolic and mitochondria fractions of rat liver (Shieh and Chen, 1993; Reichel *et al.*, 1995) and a cDNA clone coding for the rat liver cytosolic enzyme has recently been isolated and the protein expressed in *E. coli* (Reichel *et al.*, 1997). Hydrolysis of either of the acyl-CoA thioesters results in the liberation of the free acid.

An alternative pathway to the hydrolysis of the acyl-CoA thioesters is transfer of the acyl group into glycerolipids; for example, both ibuprofen and fenoprofen have been shown to be incorporated into adipocyte triacylglycerols, forming "hybrid" glycerides (Williams *et al.*, 1986, Sallustio *et al.*, 1988). These hybrid glycerides accumulate in adipose tissue forming "long-lasting" stores and have been shown to interfere with various aspects of lipid biochemistry and membrane function; including inhibition of endogenous lipid synthesis, disruption of lipid β-oxidation and alteration of membrane structure (Hall and Xiaotao, 1994; Mayer, 1996). The toxicological implications of these pathways for man is at present unclear, but as the formation of the acyl-CoA derivatives is essentially stereospecific for the (*R*)-2-arylpropionic acids a case may be made for those drugs which undergo inversion to be used as the single *S*-enantiomers.

Other metabolic routes

All of the 2-arylpropionic acids are excreted in the urine, mainly in the form of oxidative metabolites and as conjugates of amino acids or glucuronic acid. Although glucuronidation is the dominant metabolic pathway for most 2-arylpropionic acids, functional oxidation appears to play a more significant role in the cases of ibuprofen and flurbiprofen. Ibuprofen is oxidised to form two major metabolites, hydroxy- and carboxyibuprofen in man, accounting for approximately 26 and 35 % of the administered dose excreted in urine (Mills *et al.*, 1973). The observation that both the oxidation products were dextrorotatory, irrespective of the stereochemical form of

ibuprofen administered, i.e. either of the enantiomers or the racemate, ultimately resulted in the discovery of the chiral inversion reaction (Mills *et al.*, 1973; Hutt and Caldwell, 1983). In man, flurbiprofen undergoes aromatic oxidation to yield 4'-hydroxyflurbiprofen, the major metabolite accounting for *ca.* 48 % of the dose recovered in urine, and 3',4'-dihydroxyflurbiprofen followed by methylation to 3'-hydroxy-4'-methoxyflurbiprofen (Szpunar *et al.*, 1987). Mean urinary recovery studies of (*R*)- and (*S*)-flurbiprofen and its 4'-hydroxy metabolite suggested a lack of stereoselectivity of the oxidative reaction (Small *et al.*, 1990). However, another investigation revealed that the urinary metabolites of flurbiprofen had an *S/R* ratio of 0.80, which prompted the authors to suggest that enantioselectivity exists in the oxidation and conjugation reactions (Knadler and Hall, 1989).

There is increasing evidence that cytochrome P450 2C9 (CYP 2C9) is the important metabolising enzyme in the oxidative metabolism of 2-arylpropionic acids. Sulphaphenazole and/or tolbutamide inhibition along with kinetic studies utilising recombinant CYP 2C9 indicate that this isoform is probably the major contributor to the human hepatic 2- and 3- hydroxylation of (*R*)- and (*S*)-ibuprofen and solely responsible for the 4'-hydroxylation of (*R*)- and (*S*)-flurbiprofen (Mancy *et al.*, 1995; Tracy *et al.*, 1995; Hamman *et al.*, 1997). Similar approaches have demonstrated that CYP 2C9 is involved in *O*-demethylation of (*S*)-naproxen and hydroxylation of the thiophene ring of suprofen (Mancy *et al.*, 1995; Miners *et al.*, 1996; Rodrigues *et al.*, 1996).

The 2-arylpropionic acids and their major oxidative metabolites undergo varying degrees of phase II glucuronidation. Principally these compounds form 1-*O*-β-acyl (ester) conjugates with optically active D-glucuronic acid from the glucuronosyl donor, uridine diphosphoglucuronic acid (UDPGA). This transfer is catalysed by a microsomal enzyme, UDP-glucuronosyltransferase. *In vitro* investigations using microsomes from rabbit, rhesus monkey and human liver described enantioselective glucuronidation of ibuprofen, benoxaprofen and naproxen in favour of the *S*-enantiomer (El Mouelhi *et al.*, 1987). Additionally, preferential glucuronidation of the *R*-enantiomer of flunoxaprofen, flurbiprofen, indoprofen, pirprofen, benoxaprofen, carprofen and cicloprofen has been observed using rat liver microsomes (Hayball, 1995). Furthermore, *in vitro* studies indicate that (*R*)-flurbiprofen is a better substrate that its antipode for glucuronidation in humans (Hamdoune *et al.*, 1995).

The acyl glucuronides formed are intrinsically chemically reactive species and in the presence of plasma proteins may hydrolyse and release the parent molecule into the systemic circulation, this may also occur in the bladder and this reversible process is referred to as futile recycling (Meffin *et al.*, 1983). It is also important to note that this reaction can also display enantioselectivity, Knadler and Hall (1991) reported preferential hydrolysis of the conjugates of (*S*)-flurbiprofen in both plasma and albumin solutions and relatively little (nonstereoselective) conjugate hydrolysis in buffer alone. In conditions of renal dysfunction, accumulation of glucuronides occurs due to reduced elimination and the effect of futile recycling is to regenerate the free drug and maybe also to amplify chiral inversion for those drugs subject to this process, leading to an accumulation of the active *S*-enantiomer (Hayball, 1995). Consistent with this systemic cycling hypothesis, is the selective accumulation of (*S*)-ketoprofen and its glucuronide in chronic haemodialysis patients (Grubb *et al.*, 1999). Acyl glucuronides may also undergo intramolecular rearrangement by acyl group migration to yield positional isomers which remain sensitive to chemical hydrolysis but are resistant to enzymatic hydrolysis by β-glucuronidase (Faed, 1984; Caldwell and Hutt, 1986). In addition to hydrolysis and isomerisation reactions, the electrophilic character of acyl glucuronides enables them to covalently bind to appropriate nucleophilic sites on biological macromolecules and potentially cause immunotoxicological responses (Faed, 1984, Spahn-Lannguth and Benet, 1992). The formation of such complexes has been observed *in vitro* for benoxaprofen (Van Breemen and Fenselau, 1985), fenoprofen (Volland *et al.*, 1991), ketoprofen (Dubois *et al.*, 1993b) and carprofen (Iwakawa *et al.*, 1990).

Renal excretion

Renal excretion of the unchanged species is typically a minor elimination pathway for 2-arylpropionic acids, which is reflective of their high lipohilicity (Williams *et al.*, 1993).

1.5. Enantiospecific bioanalysis of 2-arylpropionic acids

Analytical methodology suitable for both the determination of enantiomeric purity and stereochemical composition in biological fluids is obviously an essential pre-

requisite for evaluation of the eudismic ratio and the pharmacokinetic profile of a drug administered as a racemate. A number of analytical techniques provide stereochemical information (Table 1.2), but those most appropriate for the bioanalyst are based on separation sciences since they provide a means of discriminating between the analyte and the complex, often chiral, biological media. Enantioselective separation methods based on gas chromatography are rather limited due to the need for high column temperatures, volatility of analyte and the non-feasibility of large-scale preparative separations. In recent years, advances in capillary electrophoresis and supercritical fluid chromatography have seen increasing applications to enantiomeric resolution, although as yet there are few reports of their use in stereospecific bioanalysis (Lynam and Nicolas, 1993; Wilson, 1994; Ward and Ward, 1997; Hutt and Patel, 1998; Soo *et al.*, 1999). The most frequently used technique is high-performance liquid chromatography, with chiral resolution achieved using either a **indirect** or **direct** approach. Both approaches will be described below, including brief details of their application in the enantiomeric analysis of 2-arylpropionic acids.

Table 1.2: Common enantiospecific analytical methodologies (adapted from Hutt *et al.*, 1994; Hutt and Patel, 1998).

Approach	Method *
Physical	polarimetry, circular dichroism
	pseudoracemic mixture of enantiomers labelled with either stable or radioisotope
	NMR spectroscopy in the presence of chiral shift reagent, solvating or derivatising agents
Separation science	chromatographic : GC, HPLC, SFC
	capillary electrophoresis : FSCE, MEKC
Biological	enantiospecific radioimmunoassay
	radioreceptor assays

** Key: NMR= nuclear magnetic resonance, GC= gas chromatography, HPLC= high performance liquid chromatography, FSCE= free solution capillary electrophoresis, MEKC= micellar electrokinetic chromatography.*

Indirect approach

This approach is based on derivatization of the analyte enantiomers with a homochiral derivatizing agent (HDCA) to produce stable diastereoisomers. The formed derivatives, owing to their different physicochemical properties, can be separated using

conventional chromatographic stationary phases. The advantage of this approach is that appropriate selection of the HCDA in relation to the available detection systems, offers the opportunity to enhance analytical sensitivity, specificity and selectivity. This approach is also favoured since achiral stationary phases are utilised and these are often more robust, stable, efficient and economical than chiral stationary phases (CSPs). However, this approach does have a number of limitations including: presence of optical impurity in the HCDA, racemisation of the analyte or HCDA during derivatization, stereoselective derivatization (i.e. kinetic resolution), low product yield and differential detector responses to the two diastereoisomers. Of fundamental significance is a knowledge of the purity of the HCDA employed, since only if the HCDA is 100 % enantiomerically pure and kinetic resolution is not a factor, will the diastereomeric ratio be representative of the enantiomeric composition in the original analytical sample. Otherwise, the enantiomeric purity of the HCDA would have to be taken into consideration to avoid errors in the calculated values as detailed by Allenmark (1988), Wozniak *et al.* (1991) and Hutt *et al.* (1994).

It is obviously essential for this approach to be applicable that the analyte(s) under investigation contain a suitable functionality which may undergo derivatization. In the case of 2-arylpropionic acids, the carboxyl group can be readily transformed into an ester or amide moiety (Figure 1.20). Methods involving the formation of esters using (*S*)-2-octanol (Lee *et al.*, 1984), (*R*)-2,2,2-trifluoro-1-(9-anthryl)ethanol (Zhao *et al.*, 1994) and (1*R*,2*S*,5*R*)-menthol (Chen and Chen, 1994) as HCDAs have been reported for ibuprofen, and (*S*)-octanol has also been applied in a method for the determination of the enantiomeric purity of naproxen (Johnson *et al.*, 1979). However, this approach is generally not favoured due to the instability of esters in comparison to amides (Testa, 1986) and consequently interest shifted towards chiral amines as HCDAs.

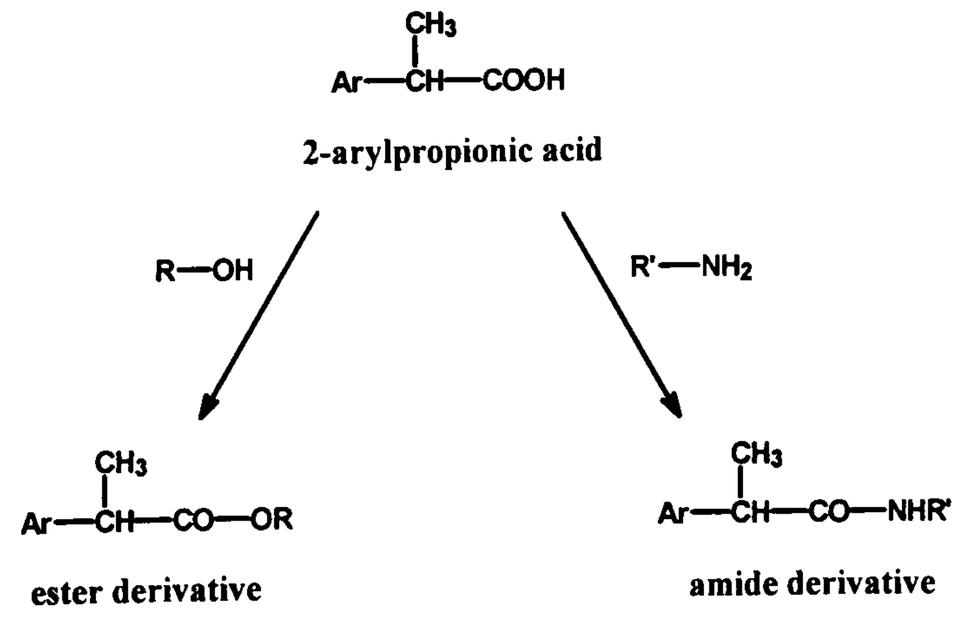


Figure 1.20: Formation of ester and amide derivatives of 2-arylpropionic acids.

Numerous chiral amines have been utilised as HCDAs, the most widely used being (*R*)- and (*S*)-amphetamine, L-leucinamide, (*R*)- and (*S*)-1-phenylethylamine and (*R*)- and (*S*)-1-(naphthen-1-yl)ethylamine (Davies, 1997). These HCDAs are readily available in very high and reliable enantiomeric purity. Furthermore, derivatization of the 2-arylpropionic acid with 1-(naphthen-1-yl)ethylamine, which is a strong fluorophore, has the added advantage of enhancing detection. The formation of the amide derivatives requires activation of the carboxyl group of the 2-arylpropionic acid to enable coupling with the chiral amine, the four main chemical activation approaches are illustrated in Figure 1.21.

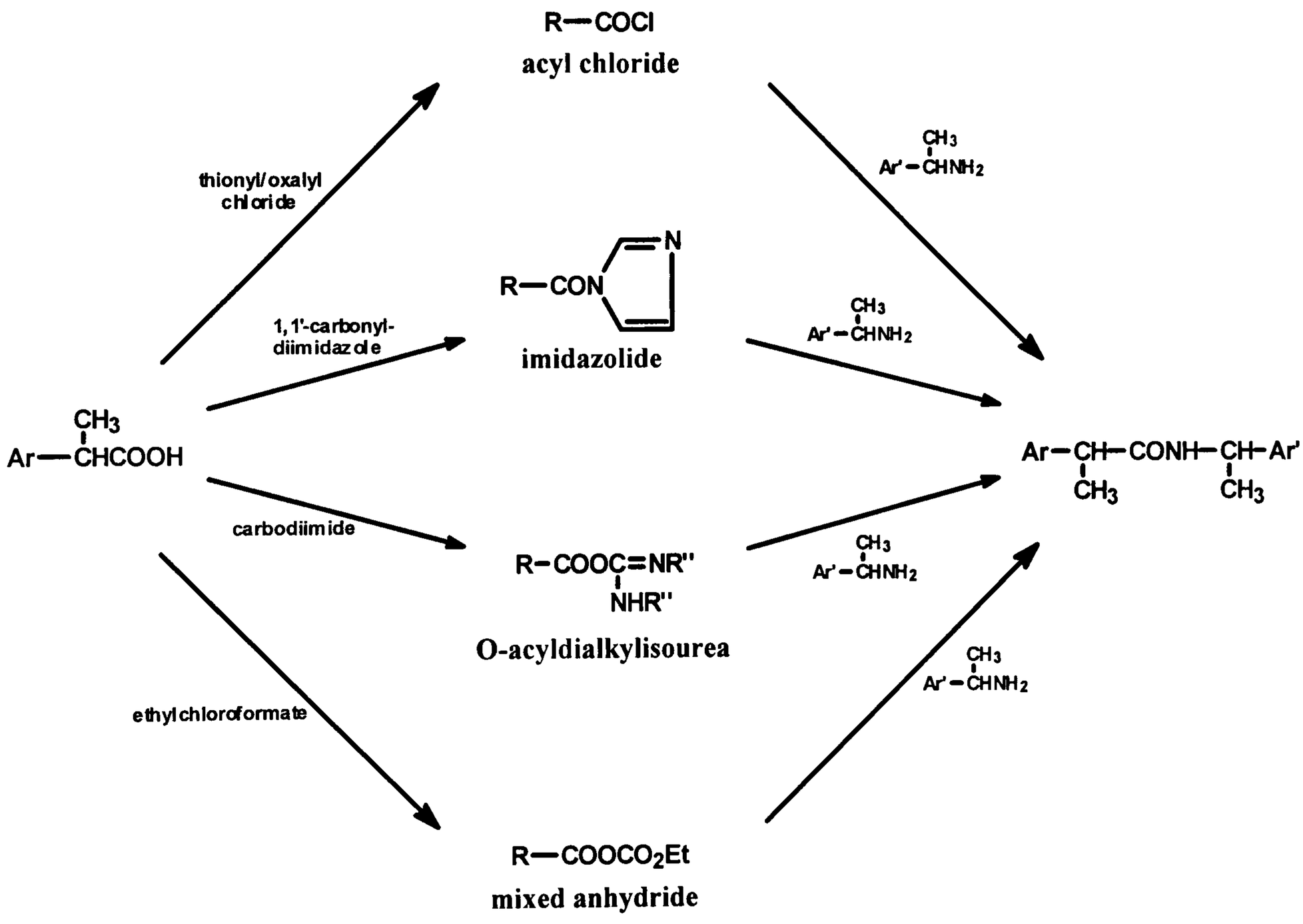


Figure 1.21: Amide formation pathways for 2-arylpropionic acids.

The early GC assays of ibuprofen (Brooks and Gilbert, 1974), and indoprofen (Tosolini *et al.*, 1974) were based on reaction with thionyl chloride to form the corresponding acyl chloride before reaction with either (*R*)- or (*S*)-1-phenylethylamine. However, this activation agent has been associated with side reactions (Kaiser *et al.*, 1976) and so its use has been superseded by other reagents. A popular activating agent is 1,1'-carbonyldiimidazole which coverts the carboxyl moiety into the corresponding imidazolide derivative (Maitre *et al.*, 1984; Young *et al.*, 1986; Rudy *et al.*, 1990). The

major drawbacks with use of this activation pathway are uncertainty of the optimal coupling conditions, which often require high temperatures; and the possibility of forming of *N,N*-disubstituted urea derivatives (Hutt, 1990; Davies, 1997). Activation using carbodiimides has been employed for the enantiomeric analysis of pirpofen, carprofen and 2-phenylpropionic acid (Hutt *et al.*, 1986) and ibuprofen (Avgerinos and Hutt, 1987; Tan *et al.*, 1997a); but this approach tends to require long derivatization times of up to two hours (Hutt *et al.*, 1986). The fourth method of activation involves the use of ethylchloroformate in the presence of an organic base, which has been widely applied for coupling 2-arylpropionic acids to various chiral amines (Bjorkman, 1985; Spahn, 1987; Palylyk and Jamali, 1991; Wright *et al.*, 1992; Lemko *et al.*, 1993). This approach is favoured for the short reaction times, which are typically between two to three minutes. The suitability of ethylchloroformate in combination with L-leucinamide as the HCDA for the resolution of 2-arylpropionic acid derivatives was reported to be a "generally applicable" method (Spahn, 1987). However, subsequent studies have show that the use of the mixed anhydride method was associated with racemization of tiaprofenic acid (Hutt *et al.*, 1994), ibuprofen (Ahn *et al.*, 1994), ketoprofen and flurbiprofen (Wright and Jamali, 1993). The extent of racemization seems to be influenced by the concentration of chloroformate (Wright and Jamali, 1993), the nature of the organic base (Hutt *et al.*, 1994) and the solvent (Ahn *et al.*, 1994).

Direct approach

The direct approach to enantiomeric analysis involves the formation of transient diastereomeric complexes via interaction of the chiral analyte(s) with the chromatographic sorbent support, i.e. chiral stationary phase (CSP) or a homochiral solvent, i.e. chiral mobile phase additive (CMPA).

i) Chiral stationary phases

The mechanism of enantiomeric resolution using CSPs is generally attributed to the "three-point" fit model of Dalgliesh (1952). For chiral recognition, one of the enantiomers of the analyte must be involved in three simultaneous interactions, one of which is stereochemically dependent, with complementary sites on the CSP and the antipode may only interact at two such sites. Consequently, the enantiomer forming the more stable diastereomeric complex will be retained longer by the CSP. The types of

interactions involved depend on the nature of the CSP and may include hydrogen bonding, dipole-dipole interactions, charge transfer complexes, inclusion complex formation, hydrophobic interactions and steric repulsion (Wainer, 1987). It is important to note, that the three interactions do not have to be attractive forces for discrimination to occur, e.g. one may be steric repulsion leading to destabilisation of the complex and in this case the enantiomer with the three-point interaction will be eluted from the column first (Wainer, 1987; Hutt *et al.*, 1994). The HPLC CSPs have been classified by Wainer (1987) into five groups based on a combination of their resolution mechanism and structural features of the chiral selector (Table 1.3). However, some of the comparatively modern chiral selectors, such as chiral crown ether phases and the macrocyclic antibiotic phases, do not directly fit these categories.

Table 1.3: Classification of chiral stationary phases (adapted from Wainer, 1987).

Type	Phase	Interactions	Examples
I	Brush type	hydrogen bonding π-donor /acceptor dipole-dipole stacking steric	(*R*)-*N*-(3,5-dinitrobenzoyl)-phenylglycine urea derivatives ergoline skeleton
II	Derivatized polysaccharide	hydrogen bonding π-donor /acceptor dipole-dipole stacking partial inclusion complexes	synthetic ether, ester or carbamate derivatives of cellulose (e.g. Chiralcel OD) or amylose (e.g. Chiralpak AD)
III	Inclusion complex	hydrogen bonding inclusion complexes	α, β, γ -cyclodextrins
IV	Chiral ligand-exchange	reversible formation of complexes between metal ions and chiral complexing agents	amino acids bound to silica via a 3-glycicloxypropyl spacer group
V	protein	hydrophobic polar steric	α_1-acid glycoprotein bovine/human serum albumin ovomucoid riboflavin binding proteins

The advantage of using CSPs is that, in most situations, pre-column derivatization of the analyte(s) is not necessary. However, should derivatization be required for any purpose; e.g. to enhance detection, to introduce an aromatic moiety complementary to

the aromatic function in the CSP or to attenuate the polarity of the functional group in the analyte to reduce individual polar interactions; the derivatizing agent need not be chiral. Therefore, problems of enantiomeric purity, racemization and differential detector responses are avoided (Hutt *et al.*, 1994).

However, the application of CSPs in quantitative bioanalysis methods has a number of limitations. CSPs do not have the versatility of conventional stationary phases since their range of applications are often limited, chromatographic conditions tend to be restricted and peak asymmetry can mean higher limits of detection. Furthermore, the presence of endogenous contaminants, frequently present in samples of biological origin, may have marked effect on both analyte retention and resolution and may also degrade a potentially expensive CSP (Ducharme *et al.*, 1996; Hutt and Patel, 1998). Additional problems may arise, as while the enantioselectivity of a CSP may be satisfactory, the chemical selectivity may be relatively poor such that structurally-related metabolic products may co-elute with the drug enantiomers (Hutt and Patel, 1998). One approach to overcome this problem is to use sequential achiral-chiral chromatography. The total drug or metabolite content of a sample is determined using the achiral phase and the mobile phase eluate containing the appropriate analyte is collected for subsequent enantiomeric analysis on the CSP (Hutt and Patel, 1998).

One of the initial reports of the use of a CSP for the enantiomeric resolution of 2-arylpropionic acids employed a Brush-type "Pirkle" column (Wainer and Doyle, 1984) However, optimal separation on this (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine column required pre-column derivatization of the drug with 1-naphthenemethylamine. A chiral recognition model was developed, based on these experiments, which suggested involvement of stacking of amide dipoles, π–π interactions between aromatic groups of the dinitrophenyl ring on the CSP and the drug, together with hydrogen bonding. Resolution of flurbiprofen enantiomers using a similar approach was consistent with this chiral recognition model (McDaniel and Snider, 1987). However, the validity of this model has been challenged by a proposed model of antiparellel amide stacking with hydrogen bonding, π–π interactions between the naphthyl group of the analyte derivative and the dinitrophenyl ring of the CSP and between the analyte aromatic ring and the unsubstituted phenyl group of the CSP (Nicoll-Griffith, 1987). The use of Pirkle-type phases for the bioanalysis of 2-arylpropionic acids has been reported by Crowther *et al.* (1984), Sioufi *et al.* (1987) and Nicoll-Griffith *et al.* (1988) and in all

cases pre-column derivatization was essential. Direct separation of several 2-aryl-propionic acids by HPLC with amide and urea derivatives bonded to silica gel as CSPs has also been described (Oi *et al.*, 1995). In addition, the use of an ergot alkaloid based stationary phase, packed in microbore columns, for the resolution of the enantiomers of several NSAIDs has potential for application in bioanalysis (Castellani *et al.*, 1994, Olsovska *et al.*, 1999).

Numerous polysaccharide CSPs are available with the tris(3,5-dimethylphenyl carbamate) derivatives of cellulose (Chiralcel OD) and amylose (Chiralpak AD) appearing to be the most versatile (Okamoto and Yashima, 1998). Analyte interactions with these phases are primarily via attractive forces and formation of inclusion complexes, with stereoselectivity based upon steric fit of the analyte into the chiral cavities. The direct enantiomeric resolution of flurbiprofen, tiaprofenic acid and ketoprofen has been reported for the Chiralpak AD phase; however, ibuprofen enantiomers could only be resolved as their methyl esters (Okamoto *et al.*, 1989). This CSP has also more recently been applied in a sequential achiral-chiral method for the analysis of ibuprofen metabolites in urine (Tan *et al.*, 1997b). The main disadvantage of these phases is the restricted choice of mobile phases that may be selected since they are incompatible with chlorinated and aqueous phases. Novel phases are being developed such that the polysaccharide is chemically bonded rather than coated onto the support, which will enable the use of more polar solvents (Enomoto *et al.*, 1996).

A β-cyclodextrin bonded phase, i.e. a type III CSP, where separation is based on the formation of inclusion complexes has been successfully employed to resolve underivatized ibuprofen enantiomers following isolation from biological fluids (Giesslinger *et al.*, 1989). However, enantiomeric resolution was not complete and the phase displayed temperature sensitivity and batch-to-batch variability.

Several direct resolutions of underivatized 2-arylpropionic acids have been described using protein affinity CSPs. These were based on either bovine serum albumin (Allenmark and Andersson, 1989), human serum albumin (Nocter *et al.*, 1991), ovomucoid (Miwa *et al.*, 1987; Haginaka *et al.*, 1993), α_1-acid glycoprotein (Hermansson and Eriksson, 1986), avidin (Oda *et al.*, 1991) or egg yolk riboflavin binding protein (Massolini *et al.*, 1995; De Lorenzi and Massolini, 1999). The main advantages of protein phases for bioanalytical applications are that they are used in the reversed-phase mode and show considerable versatility. However, they tend to have limited analyte capacity, may be readily overloaded and enantiomeric resolution can be

extremely sensitive to subtle modifications in mobile phase composition (Hutt and Patel, 1998).

ii) Chiral mobile phase additives

The use of a CMPA enables enantiomeric resolution if the formed labile diastereomeric complexes differ in their distribution between the mobile phase and the achiral stationary phase. The main advantages of this approach are the use of conventional efficient achiral phases and the limited cost of most CMPAs. Possible disadvantages include the fact that some CMPAs possess chromophores and thus impose a limitation on their usefulness and removal of the CMPA from the eluate may be a problem if the analyte is required in the pure form (Armstrong, 1987).

Enantiomeric resolution of naproxen and other 2-arylpropionic acids has been investigated using quinine as a CMPA with normal-phase columns (Pettersson, 1984). Separation efficiency was dependent on the nature of the stationary phase, i.e. whether silica diol or cyano bonded phases were employed. However, the applicability of this approach for bioanalysis is limited by the presence of strong chromophores in quinine which would adversely influence analytical sensitivity. The use of β-cyclodextrin as a CMPA proved to be unsatisfactory for the resolution of ibuprofen and pirprofen (Hutt and Caldwell, 1988). However, the enantiomers of fenoprofen and ketoprofen have recently been resolved using a nonporous reversed-phase column with hydroxypropyl β-cyclodextrin as a CMPA, with the approach being subsequently developed into an assay for the determination of the enantiomeric composition of ketoprofen in serum (Ameyibor and Stewart, 1997, 1998).

1.6. Aims and objectives

NSAIDs, with the 2-arylpropionic acids being the major sub-group, are among the most widely used therapeutic agents today, with approximately 20 million prescriptions dispensed for NSAIDs per year in the UK at a cost of over £180 million (Bateman and Kennedy, 1995). However, their use also represents an important cause of morbidity and mortality and is a major drain on resources in both primary and secondary health care (Garcia Rodriguez and Jick, 1994; Langman *et al.*, 1994). Epidemiological studies have

shown that the elderly population are the most frequent users of NSAIDs and at the most at risk of adverse effects; which in part could be attributed to age-related alterations in disposition (Henry *et al.*, 1993; Garcia Rodriguez and Jick, 1994).

It is therefore the more surprising that little data is available in the literature on the pharmacokinetics of NSAIDs in this target population, with most studies performed using relatively young healthy volunteers. Furthermore, those few studies which have investigated the influence of ageing on the pharmacokinetics of NSAIDs have tended to be of deficient study design and have not considered stereochemical factors, despite the fact that the majority of NSAIDs possess a chiral centre and are marketed as racemates (Davies and Skjodt, 2000). Examination of potential age associated alterations in the disposition of NSAIDs in the elderly, particularly if such alterations are stereoselective, may provide some insight into the observed adverse reaction profile in this age group. In addition, such investigations have the potential to contribute to the more rational use of these agents in this ever increasing population sub-group.

The problems of non-stereospecific drug analysis in comparing age-related differences in pharmacokinetics following administration of racemic drugs may be illustrated by the report of Chandler *et al.* (1988) who examined the disposition of hexobarbitone. Following the administration of the racemic drug to young and elderly volunteers no differences were observed in the oral clearance of the total drug between the two groups. However, on examination of the individual enantiomers the clearance of the (-)-isomer was found to be twice as fast in the young volunteers as in the elderly group. It is also noteworthy, that the situation can be complicated by age-related gender differences in enantiomeric disposition as has been previously observed for methylphenobarbitone, see section 3.4 for further details (Hooper and Qing, 1990). Nevertheless, in some cases age-related alterations in disposition may not display stereoselectivity, for example the elderly have a lower oral clearance for both enantiomers of propranolol than the young with no effect on the enantiomeric composition in serum (Zhou *et al.*, 1992).

It was therefore proposed to examine the enantioselective disposition and pharmacokinetics of two 2-arylpropionic acid drugs, namely ibuprofen and flurbiprofen, following the administration of the racemic form of the drugs to healthy young and elderly volunteers. These two drugs although structurally related were selected as they have considerably different pharmacological properties, and thus it is possible that

advancing age has a differential influence. Differences in the pharmacodynamic profile between the two drugs is not only likely to be due to their *S*-enantiomers displaying different COX-1 to COX-2 selectivities, but also due to the varied role played by their respective *R*-enantiomers. For example, with ibuprofen the *R*-enantiomer is essentially a pro-drug which contributes to anti-inflammatory activity, not only by significant metabolic inversion to (*S*)-ibuprofen but also via inhibition of induction of COX-2 mediated by (*R*)-ibuprofenoyl-CoA thioester (Neupert *et al.*, 1997). In contrast, the *R*-enantiomer of flurbiprofen does not undergo unidirectional inversion in man but may contribute therapeutically via central antinociceptive effects (Brune *et al.*, 1992). Differences in enantioselective disposition between ibuprofen and flurbiprofen, as would be expected, most probably stem from the contrasting significance of the chiral inversion process; but other important influencing factors are likely to be differences in stereoselectivity for protein binding and/or for metabolism via oxidation and conjugation pathways.

In order to examine the stereoselectivity of the disposition of ibuprofen and flurbiprofen validated HPLC analytical methods for monitoring the enantiomeric composition of the drugs, and their metabolites, in biological fluids are an essential prerequisite. Appropriate analytical methodologies for the enantiospecific analysis of ibuprofen in serum and urine and for the stereospecific analysis of its two major metabolites, hydroxy and carboxyibuprofen, in urine have been previously developed within the laboratory (Tan *et al.*, 1997a, 1997b); the applicability of these methods for clinical studies will be assessed. Numerous methods have been published which describe the enantiospecific bioanalysis of flurbiprofen, but only one of these also considers the determination of the enantiomeric composition of its two major metabolites, 4'-hydroxy and 3'-hydroxy-4'-methoxyflurbiprofen (Knadler and Hall, 1989). However, this particular methodology is unable to analyse all three analytes in a single chromatographic run and was thus deemed inappropriate for routine use. Therefore various chromatographic approaches, both direct and indirect, will be investigated for their ability to simultaneously separate and resolve the enantiomers of flurbiprofen and its metabolites. Subsequently, the chromatographic system which displays the desired separation profile will be adapted into methodologies suitable for the determination of the enantiomeric composition of flurbiprofen and the two metabolites in urine and serum. As authentic reference samples of the enantiomers of the two flurbiprofen metabolites are not available, an attempt will be made to isolate the

individual isomers using semi-preparative chromatography and assignment of stereochemistry by chiroptical spectroscopic methods will be performed to establish the chromatographic elution order.

Furthermore, the influence of age on the pharmacodynamic properties of ibuprofen following the administration of the racemate have not been reported previously. For this purpose, inhibitory activity of the drug on cyclooxygenase function will be monitored in blood samples obtained from both young and elderly volunteers during the pharmacokinetic study by measurement of thromboxane A_2 generation and function. These latter investigations will be performed in association with the Department of Pharmacology, King's College London.

CHAPTER 2 :

Stereospecific analysis of ibuprofen and its two major metabolites in biological fluids

2.1. Introduction

Ibuprofen undergoes extensive unidirectional chiral inversion in humans from the relatively inactive *R*-enantiomer to its active *S*-antipode as outlined in Chapter One (Mills *et al.*, 1973; Wechter *et al.*, 1974; Kaiser *et al.*, 1976). In addition to inversion the metabolism of ibuprofen involves conjugation with glucuronic acid and oxidation of the isobutyl group at the 2- and 3- positions to yield hydroxy and carboxyibuprofen respectively (Figure 2.1; Mills *et al.*, 1973). Following oral administration of racemic ibuprofen, approximately 60% of the dose is recovered in a 24 hour urine collection as these metabolites, both free and conjugated with glucuronic acid (Mills *et al.*, 1973). The observation that both the oxidation products were dextrorotatory, regardless of the stereochemical form of ibuprofen administered, i.e. the racemate or either enantiomer, ultimately resulted in the discovery of the metabolic chiral inversion reaction (Mills *et al.*, 1973; Hutt and Caldwell, 1983; Caldwell *et al.*, 1988a).

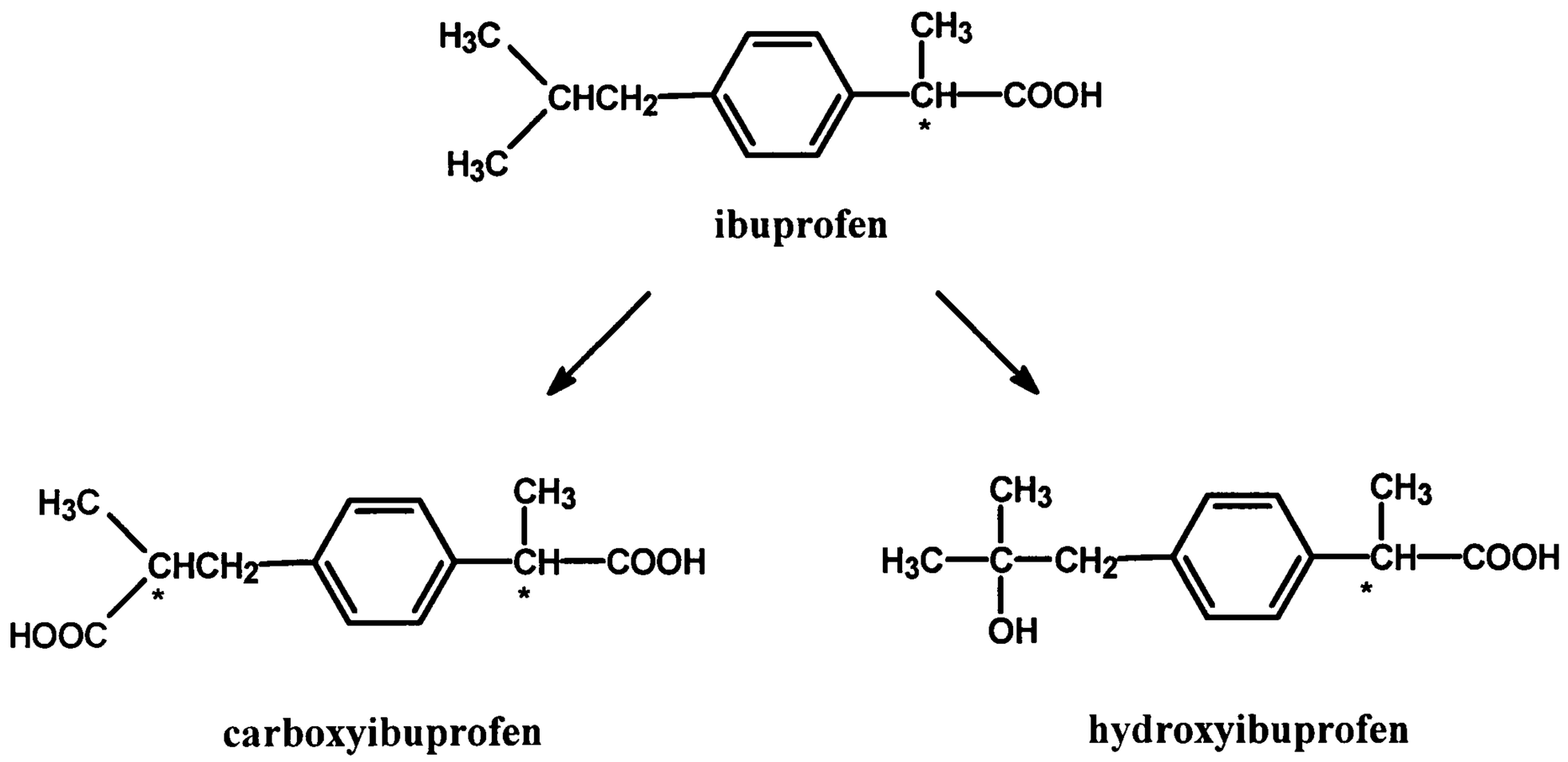


Figure 2.1: Major oxidative metabolic pathways of ibuprofen (* denotes chiral centre).

The considerable interest in the chiral inversion process and stereochemical aspects of ibuprofen disposition has led to the publication of a vast number of enantiospecific assay methods, based on both direct and indirect approaches, for this first-generation and important 2-arylpropionic acid drug. The direct approach, utilising α_1-acid glycoprotein phases (Menzel-Soglowek *et al.*, 1990; Geisslinger *et al.*, 1990; Pettersson and Olson, 1991; De Vries *et al.*, 1994) and β-cyclodextrin phases

(Geisslinger *et al.*, 1989; Naidong and Lee, 1994) have been employed for the determination of ibuprofen enantiomers in plasma and urine. Other chiral stationary phases (CSPs) used for the enantiospecific analysis of ibuprofen include Pirkle-type (Wainer and Doyle, 1984; Nicoll-Griffith *et al.*, 1988, 1993; Li *et al.*, 1993) and derivatized polysaccharide phases (Okamoto *et al.*, 1989; Van Overbeke *et al.*, 1994); these methods however required pre-column derivatization of ibuprofen to obtain acceptable resolution.

Some indirect methods for the determination of ibuprofen enantiomers have employed alcohols as homo-chiral derivatizing reagents (HCDAs), e.g. (*S*)-octanol (Lee *et al.*, 1984), (*R*)-2,2,2-trifluoro-1-(9-anthryl)ethanol (Zhao *et al.*, 1994) and (1*R*,2*S*,5*R*)-menthol (Chen *et al.*, 1991; Chen and Chen, 1994); but this approach is generally not favoured due to the labile nature of the ester derivatives (Testa, 1986). Moreover, indirect methods have been based on the formation of relatively stable diastereomeric amides after derivatization with optically pure chiral amines such as L-leucinamide (Foster and Jamali, 1987; Pehourcq *et al.*, 1995), (*R*)- and (*S*)-1-phenylethylamine (Rudy *et al.*, 1990; Wright *et al.*, 1992), (*R*)- and (*S*)-amphetamine (Singh *et al.*, 1986; Jack *et al.*, 1992), (*R*)- and (*S*)-1-(naphthen-1-yl)ethylamine (Avgerinos and Hutt, 1987; Mehvar *et al.*, 1988; Lemko *et al.*, 1993; Lau, 1996) and (-)-2-[4-(1-aminoethyl)phenyl]-6-methoxybenzoxazole (Kondo *et al.*, 1994). These chiral amines often have strong chromophores or fluorophores which aid the analysis by increasing assay sensitivity. Recently, a reliable, sensitive enantiospecific assay for the determination of ibuprofen in serum and urine based on reversed phase high-performance liquid chromatography (HPLC) has been developed within our laboratory (Tan *et al.*, 1997a). The use of (*R*)-1-(naphthen-1-yl)ethylamine as a chiral derivatizing agent in combination with fluorescence detection enabled the approach to be adapted for the determination of the free drug enantiomer concentrations in serum following equilibrium dialysis (Tan *et al.*, 1997a).

In comparison to the parent drug there are relatively few reports concerned with the determination of the stereochemical composition of the two major oxidation products. The enantiomers of hydroxyibuprofen have been resolved by both gas chromatography and HPLC following derivatization with the HCDAs 1-phenyl-ethylamine (Kaiser *et al.*, 1976; Young *et al.*, 1986; Baille *et al.*, 1989; Rudy *et al.*, 1990) and (1*R*,2*S*,5*R*)-menthol (Chen *et al.*, 1991; Chen and Chen, 1994) and using a (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine CSP (Nicoll-Griffith *et al.*, 1988). However,

formation of carboxyibuprofen results in the introduction of a second chiral centre into the molecule (Figure 2.1) and the chromatographic resolution of the four stereoisomers, via either derivatization using an HCDA or CSP, has proved to be problematical. The only successful chromatographic resolution was that of Rudy *et al.* (1990) which was based on the indirect approach following derivatization with (*S*)-1-phenylethylamine and HPLC using a C_8 stationary phase. Due to the lack of stereochemically defined authentic standards, the assignment of the chromatographic elution order of the carboxyibuprofen stereoisomers was based on that published by Kaiser *et al.* (1976), which was itself empirical. However, more recently within our laboratory, Tan *et al.* (1997c) were able to resolve the stereoisomers of carboxyibuprofen using the derivatized amylose CSP, amylose tris (3,5-dimethylphenylcarbamate) (Chiralpak AD) and determine their elution order by a combination of both metabolic and synthetic approaches. Subsequently, the above methodology was adapted to develop a sequential achiral-chiral chromatographic technique for the determination of the stereochemical composition of carboxy and hydroxyibuprofen in urine (Tan *et al.*, 1997b). Total metabolite concentrations are determined by achiral normal-phase chromatography, followed by collection of the column eluates containing the individual metabolites prior to the determination of their stereochemical composition using the CSP.

The "in-house" development of novel HPLC methods for the enantiospecific analysis of ibuprofen in serum and urine and for the stereospecific analysis of the two major metabolites in urine have been outlined above. The objectives of this chapter are to describe in detail the analytical methodology employed, as well as any modifications made to the published methods, and to describe the validation experiments performed prior to their application in clinical investigations (Chapter 3).

Carboxyibuprofen is chemically designated as 2-[4-(2-carboxypropyl)phenyl] propionic acid and thus both chiral centres are indicated as being in the 2-position of the two side chains. In this thesis, the chiral centre introduced by metabolic oxidation of the isobutyl group of ibuprofen is indicated as the 2'-position, whereas the original chiral centre in the propionic acid moiety is indicated as the 2-position.

2.2. Experimental

2.2.1 Chemicals and reagents

Acetonitrile, dichloromethane, ethanol, ethyl acetate, hexane and isopropanol (HPLC grade) were purchased from Rathburn (Walkerburn, UK). Sodium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, trifluoroacetic acid (TFA), diethylether (Analar grade), and other solvents were purchased from BDH (Poole, Dorset, UK). Human serum albumin (type Fraction V, HSA), 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride (CDI), (*R*)-1-(naphthen-1-yl)ethyl-amine ((*R*)-NEA) and 4-chlorophenoxyacetic acid were purchased from Sigma Chemicals (Poole, Dorset, UK). 1-Hydroxybenzotriazole (HOBT) was purchased from Fluka Chemicals (Poole, Dorset, UK). (*R,S*)-, (*R*)-, (*S*)-Ibuprofen, (*R,S*)-hydroxy-ibuprofen and (*R,S*)-flurbiprofen were the generous gifts of Boots Company PLC (Nottingham, UK). "Racemic" carboxyibuprofen was synthesised as described previously (Tan *et al.*, 1997c). Solid phase extraction (SPE) silica cartridges (100 mg) were prepared using Merck silica gel (40-63 μm) obtained from BDH (Poole, Dorset, UK) and empty Bond-elut SPE cartridges and frits purchased from Anachem Ltd. (Luton, Beds, UK). The SPE cartridges were activated by elution with methanol (1 ml) and subsequent drying at 100°C for 1 hour prior to use. Phosphate buffered saline (pH 7.4; PBS) was prepared with 0.067 M disodium hydrogen phosphate, 0.067 M sodium dihydrogen phosphate and sodium chloride. Dialysis membranes (Spectropor 2, molecular weight cut-off 12,000-14,000) were obtained from Pierce and Warriner (Chester, UK). The membranes were cut into discs (diameter, 3 cm), washed with purified water and then soaked in PBS overnight. Protein Assay reagents based on the Coomasie blue method were obtained from Bio-Rad (Herts,UK). Radiolabelled [^{14}C]-ibuprofen with specific activity of 21.6 mCi g^{-1} was generously donated by Knoll Pharmaceuticals (Nottingham, UK). The liquid scintillation cocktail used was Quickscint flow 302 (Zinsser Analytical, Berkshire, UK).

2.2.2 Chromatographic columns

The C_{18} column, a Waters Resolve C_{18} column (150 x 3.9 mm, 5 μm), was obtained from Anachem Ltd. (Luton, Beds., UK); and used in conjunction with a

refillable guard column (10 x 2.1 mm) packed with pellicular C_{18} (40-60 μm) both were obtained from Alltech Associates (Carnforth, Lancs., UK). The normal-phase column, a Partisil silica column (250 x 4.6 mm, 5 μm) was obtained from Whatman (Maidstone, UK) and was used with a refillable guard column (10 x 2.1 mm) packed with pellicular silica (40-63 μm) both were obtained from Alltech Associates (Carnforth, Lancs., UK). The chiral columns, a Chiralcel OD (cellulose tris (3,5-dimethylphenylcarbamate)) column (250 x 4.6 mm, 10 μm) and a Chiralpak AD (amylose tris (3,5-dimethylphenyl-carbamate)) column (250 x 4.6 mm, 10 μm), used with their respective guard columns (5 x 4.6 mm, 10 μm), were supplied by HPLC Technology Ltd. (Macclesfield, UK).

2.2.3 Instrumentation and apparatus

Reverse-phase HPLC was performed using an LDC Constametric 3000 pump (Stone, Staffs., UK) linked to a Merck-Hitachi spectrofluorometer (Poole, Dorset, UK) and a LDC CI-4000 computing integrator (Stone, Staffs., UK). Samples were introduced on-column using a Rheodyne 7125 injection valve (Cotati, Cal., USA) fitted with a 50 μl sample loop. Normal-phase HPLC was performed using an LDC Constametric 3000 pump linked to an LDC Spectromonitor 3000 UV detector and a LDC CI-4000 computing integrator (Stone, Staffs., UK). Samples were introduced on-column using a Rheodyne 7125 injection valve (Cotati, Cal., USA) fitted with a 100 μl sample loop. Chiral-phase HPLC was performed using an LDC Constametric 3000 pump linked to an LDC Spectromonitor 3100 UV detector and a LDC CI-4100 computing integrator (Stone, Staffs., UK). Samples were injected on column using a Perkin Elmer ISS-100 autosampler (Beaconsfield, Bucks., UK). Equilibrium dialysis cells of 2.5 ml total capacity were constructed from perspex in the Department of Pharmacy Workshop, King's College London. Liquid scintillation spectrometry was performed using an LKB 1209 Wallac Rackbeta liquid scintillation spectrometer (Milton Keynes, UK).

2.2.4 Enantiospecific analysis of ibuprofen in serum

Sample preparation

Aliquots of serum (0.5 ml) were added to 15 ml extraction tubes along with 1.0 μg (20 μl of a 0.05 mg/ml solution in acetonitrile) of (*R,S*)-flurbiprofen as internal standard. The samples were acidified by the addition of hydrochloric acid (1.0 M; 100 μl) and buffered to pH 3.8 with 1 ml of sodium phosphate buffer (pH 3.8; 1.0 M). Diethylether (3 ml) was then added and the samples mixed on a test-tube rocker for 20 minutes. Following centrifugation at 1000g for 5 minutes, the diethylether layers were transferred into clean tubes and evaporated to dryness under a stream of nitrogen at 40°C on a dry block heater.

Derivatization

The extracted samples or standards were derivatized by the addition of 100 μg each of CDI, HOBT and (*R*)-NEA (100 μl each of 1 mg/ml solutions in dichloro-methane). The contents were briefly mixed and the reaction allowed to proceed at room temperature for two hours. The reaction mixtures were then loaded onto SPE silica cartridges (100 mg) prewetted with dichloromethane and the eluate collected. The cartridges were further eluted twice with dichloromethane:acetonitrile (1 ml, 9:1 v/v) and the pooled eluate dried under nitrogen gas at 40°C. The residue was reconstituted in 100 μl mobile phase and 50 μl injected into the reverse phase HPLC system.

Chromatographic analysis

Chromatography was carried out using a Waters Resolve C_{18} (150 x 3.9 mm, 5 μm) column protected by a guard column (10 x 2.1 mm) filled with pellicular C_{18}. The mobile phase used was phosphate buffer (pH 3.5, 0.01 M):acetonitrile (50:50 v/v) at a flow rate of 1.5 ml/min at ambient temperature. Column eluate was monitored by fluorescence detection with excitation and emission wavelengths of 290 and 330 nm respectively.

Validation of the assay procedure

A stock solution of (*R,S*)-ibuprofen (10 mg/100ml) was prepared in acetonitrile. Into six separate 10 ml volumetric flasks were pipetted 0.02, 0.05, 0.1, 0.2, 1.0 and

2.0 ml aliquots of the stock solution. The solutions were evaporated gently under a stream of nitrogen and drug free serum added to the flasks q.s. 10 ml to give final solutions containing 0.1, 0.25, 0.5, 1.0, 5.0 and 10 μg/ml of each enantiomer in the respective flasks. Aliquots (0.5 ml) of these standards were transferred into different tubes and stored at -20°C. On each day of analysis one set of these tubes were analysed together with the samples. Calibration curves were constructed by plotting peak area ratios (ibuprofen enantiomer:(*S*)-flurbiprofen) against the concentration of each enantiomer and subjecting the data to linear regression analysis. The concentration of each enantiomer in serum samples was determined by comparing their respective peak area ratios to the calibration curves prepared.

The accuracy and within day variation of the assay was assessed by analysing six serum standards with the following ibuprofen enantiomer concentrations: 0.1, 1.0 and 10 μg/ml. The precision and accuracy of the assay was determined for each enantiomer by calculation of the percentage coefficient of variation (standard deviation/mean x 100) and mean percentage difference ([mean concentration-actual concentration]/ actual concentration x 100) respectively.

The between day variation of the assay was determined by analysing serum standards of the same concentrations for six consecutive days. The precision and accuracy were calculated as described above.

2.2.5 Determination of unbound concentrations of ibuprofen enantiomers in serum

Purification of [^{14}C]-ibuprofen

[^{14}C]-Ibuprofen (3 mg) was dissolved in acetonitrile (1 ml) and 50 μl aliquots were injected onto a reversed-phase Waters Resolve C_{18} column (150 x 4.6 mm, 5μm) with a mobile phase of acetonitrile:water (50:50 v/v), with the pH adjusted to 3.5 with hydrochloric acid (1.0 M), at a flow rate of 1.0 ml/min. Detection was by UV, set at 220 nm. The eluate containing ibuprofen was collected from the detector outlet into a 100 ml pear shaped flask. The combined eluate (from 20 injections) was then pooled and the solvent evaporated to half its volume under nitrogen gas at 40°C. Hexane:isopropanol (9:1 v/v; 25 ml) was added as extraction solvent and the mixture

shaken. The solvent mixture was allowed to separate and the supernatant organic layer transferred into a 50 ml pear shaped flask and evaporated to dryness under nitrogen. The chemical purity of the product was subsequently determined by redissolving a portion of the residue in mobile phase and reinjecting it into the HPLC system.

Equilibrium dialysis

The equilibrium dialysis cell consisted of two 1.25 ml chambers separated by a single layer of Spectrapor 2 dialysis membrane. Aliquots of serum (1 ml) were placed into three dialysis cells and 20 µl of purified [^{14}C]-ibuprofen (0.35 mg/ml) dissolved in HSA solution (4% w/v in PBS) added to each. Into the complimentary chambers on the opposite side of the membranes were transferred 1 ml aliquots of PBS. The cells were sealed and placed in a shaking water bath (30 strokes per minute) at 37°C for a pre-determined equilibrium time of 8 hours. After incubation, 400 µl aliquots of serum and PBS were removed from the cells and placed into separate scintillation vials along with 12.5 ml scintillation cocktail. The radioactivity, expressed as disintegrations per minute (dpm), within the buffer (dpm B) and serum (dpm P) were determined.

Sample preparation

To the dialysate buffer (2.5 ml; the contents of three cells combined) was added (*R,S*)-ibuprofen as a carrier (0.1 mg/ml in acetonitrile, 25 µl), followed by hydrochloric acid (1.0 M; 500 µl), phosphate buffer (pH 3.8, 1.0M, 2 ml), and hexane:isopropanol (9:1, v/v; 5 ml) as extraction solvent. The mixture was then extracted and solvent evaporated as described previously. The dried residue was reconstituted in 50 µl mobile phase and 30 µl injected into the Chiralcel OD CSP.

Chromatographic resolution

Enantiomeric resolution of ibuprofen was achieved using a Chiralcel OD CSP (250 x 4.6 mm, 10 µm) connected to a guard column containing similar material (50 x 4.6 mm, 10 µm). The mobile phase consisted of hexane:isopropanol (100:1.1 v/v) containing TFA (0.1% v/v) as modifier, at a flow rate of 1.0 ml/min. The UV detector was operated at 220 nm. The eluate fractions corresponding to (*R*)- and (*S*)- ibuprofen were collected between 12.0-13.2 minutes and 15.4-17.2 minutes respectively into

individual scintillation vials and the radioactivity of each fraction (dpm B_R and dpm B_S respectively) was determined.

An aliquot (200 µl) of the equilibrated serum was put through the extraction and chromatographic procedures along with the corresponding buffer sample to determine dpm P_R and dpm P_S .

Calculation of unbound fraction

The unbound fraction of (*R*)-ibuprofen ($f'u_R$) was determined as:

$$f'u_R = \frac{\text{dpm B}}{\text{dpm P}} \times \frac{\text{dpm } B_R / (\text{dpm } B_R + \text{dpm } B_S)}{\text{dpm } P_R / (\text{dpm } P_R + \text{dpm } P_S)} \qquad \text{(Eqn. 2.1)}$$

and that of (*S*)-ibuprofen ($f'u_S$) in a similar manner:

$$f'u_S = \frac{\text{dpm B}}{\text{dpm P}} \times \frac{\text{dpm } B_S / (\text{dpm } B_R + \text{dpm } B_S)}{\text{dpm } P_S / (\text{dpm } P_R + \text{dpm } P_S)} \qquad \text{(Eqn. 2.2)}$$

The unbound fractions were then corrected for volume shifts using the following equation:

$$fu = f'u.F/(f'u.F + 1 - f'u) \qquad \text{(Eqn. 2.3)}$$

where F is the ratio of the serum protein concentration post- and pre-dialysis, determined using the Coomasie blue procedure (Bio-Rad Protein Assay kit), and fu is the unbound fraction after volume shift correction (Huang, 1983). Free enantiomer concentrations were calculated by multiplying the respective corrected unbound fractions by the total (bound and unbound) serum concentrations.

Validation

Standards were prepared in drug free serum at concentrations of 5.0, 10 and 20 µg/ml of each ibuprofen enantiomer for the validation experiment. Five aliquots (i.e. 5 x

[3x1 ml]) of each of these serum standards were put through the dialysis procedure and analysed to assess the reproducibility of the method at the different concentrations.

2.2.6 Enantiospecific analysis of ibuprofen in urine

Sample preparation

Free: For the quantification of unconjugated ibuprofen, 0.5 ml aliquots of urine were used. To these samples were added 1 μg (20 μl of a 50 μg/ml solution in acetonitrile) of (*R,S*)-flurbiprofen as internal standard. The samples were then acidified by the addition of hydrochloric acid (1.0 M; 100 μl) and buffered to pH 3.8 with 1.5 ml of phosphate buffer (pH 3.8; 1.0 M). Hexane:isopropanol (9:1, v/v; 5 ml) was added and the extraction tubes were then tightly capped and mixed on a test-tube rocker for 20 minutes. Phase separation was achieved by centrifugation for 5 minutes at 1000g. The organic layer was then separated into a clean glass tube and evaporated under a gentle stream of nitrogen at 40°C on a dry heating block.

Base hydrolysis: For the determination of total concentrations (i.e., free and acyl glucuronide) of ibuprofen, 0.1 ml aliquots of urine were used. To the samples were added (*R,S*)-flurbiprofen (1 μg) followed by NaOH (1.0 M; 20 μl) for hydrolysis of the acyl glucuronic acid conjugates. The hydrolysis reaction was left to proceed for 2 hours at room temperature. Subsequently, HCl (1.0 M; 40 μl), sodium phosphate buffer (pH 3.8; 1.0 M; 200 μl) and hexane:isopropanol (9:1, v/v; 1.0 ml) were added. The samples were mixed and extraction carried out as before.

Derivatization and chromatographic conditions

The derivatization procedure and chromatographic analysis of the dried urine extracts were performed as described for the serum assay.

Validation of the assay procedure

A series of calibration standards were prepared in drug free urine at concentrations of 0.1, 0.25, 0.5, 1.0, 5.0 and 10 μg/ml of each ibuprofen enantiomer. Calibration

curves, within and between day variation were determined as described above for the serum samples.

2.2.7 Stereospecific analysis of carboxyibuprofen and hydroxyibuprofen in urine

Sample preparation

Free: For the quantification of unconjugated carboxyibuprofen and hydroxy-ibuprofen, 0.5 ml aliquots of urine were used. To these samples were added 25 μg (50 μl of a 0.5 mg/ml solution in acetonitrile) of 4-chlorophenoxyacetic acid as internal standard. The samples were then acidified by the addition of hydrochloric acid (1.0 M; 100 μl) and buffered to pH 3.8 with 1.5 ml of phosphate buffer (pH 3.8; 1.0 M). Dichloro-methane:ethyl acetate (14:1 v/v; 5 ml) was added and the extraction tubes were then tightly capped and mixed on a test-tube rocker for 20 minutes. Phase separation was achieved by centrifugation for 5 minutes at 1000g. The lower organic layer was then separated into a clean glass tube and evaporated under a gentle stream of nitrogen at 40°C on a dry heating block. The residue was reconstituted in 150 μl mobile phase and 50 μl was injected into the achiral HPLC system.

Base hydrolysis: For the determination of total concentrations (i.e., free and conjugated) of carboxyibuprofen and hydroxyibuprofen, 0.1 ml aliquots of urine were used. To the samples were added 4-chlorophenoxyacetic acid (25 μg) followed by NaOH (1.0 M; 20 μl). The hydrolysis reaction was left to proceed for 2 hours at room temperature. Subsequently, HCl (1.0 M; 40 μl), sodium phosphate buffer (pH 3.8; 1.0 M; 200 μl) and dichloromethane:ethyl acetate (14:1 v/v; 1.0 ml) were added. The samples were then extracted, dried and reconstituted as before.

Achiral chromatographic analysis and fraction collection

The achiral phase used was a Partisil silica column (250 x 4.6 mm, 5 μm) protected by a guard column (10 x 2.1 mm) filled with pellicular silica. The mobile phase consisted of hexane:ethanol (98.2:1.8 v/v) containing TFA (0.05% v/v) as modifier, at flow rate of 2.0 ml/min. The UV detector was set at 220 nm.

The column eluate from the HPLC containing carboxy (t_r, 12.6 min; fraction collected between 12.2 and 13.0 min) and hydroxyibuprofen (t_r, 15.8 min; fraction collected between 15.3 and 16.3 min) were individually collected from the detector outlet. The fractions were gently evaporated under a stream of nitrogen gas at 40°C and the residues reconstituted in 100 µl mobile phase and 50 µl aliquots subjected to chiral-phase analysis.

Chiral-phase analysis

The metabolites present in the reconstituted residues were separated into their individual isomers using a Chiralpak AD column (250 x 4.6 mm, 10 µm) connected to a guard column containing similar material (50 x 4.6 mm, 10 µm). The mobile phase consisted of hexane:ethanol (92:8 v/v) containing TFA (0.05% v/v) as modifier, run at a flow rate of 1.0 ml/min. The detection wavelength was set at 220 nm.

The enantiomeric composition of hydroxyibuprofen was determined as follows:

$$\textbf{Enantiomeric composition} = \frac{\textbf{Peak area of enantiomer}}{\textbf{Sum of peak areas for both enantiomers}} \quad \text{(Eqn. 2.4)}$$

Individual concentrations of each enantiomer were calculated by multiplying their enantiomeric composition by the corresponding metabolite concentrations obtained from achiral (normal-phase) analysis. For carboxyibuprofen, the same approach was adopted, the sum of peak areas being for all four stereoisomers.

Validation of the assay procedure

A series of calibration standards containing 10, 20, 40, 80, 160 and 320 µg/ml of each metabolite were prepared in drug free urine. On each day of analysis, 0.5 ml of these standards were analysed together with volunteer samples. Calibration curves were constructed based on the data obtained following achiral analysis. The accuracy and within day variation for each metabolite was determined by analysing the calibration samples of 10, 80 and 320 µg/ml concentrations repeatedly (n = 6). Between day variation was determined by analysing the same standards over six separate days.

The HPLC eluate from the above validation experiments were collected and subjected to chiral-phase analysis to establish whether the approach could determine accurate stereochemical compositions over a wide range of concentrations.

2.3. Results and Discussion

2.3.1 Enantiospecific analysis of ibuprofen in serum

The assay procedure used to determine the concentrations of ibuprofen enantiomers in serum was based upon the reversed-phase HPLC resolution of the fluorescent diastereomeric amides formed on reaction with (*R*)-NEA (Tan *et al.*, 1997a). The derivatization procedure is based on that original developed and optimised by Avgerinos and Hutt (1987), with derivatization of the carboxyl group being carried out using the carbodiimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (CDI), and 1-hydroxybenzotriazole (HOBT) as coupling agents (Figure 2.2). Although the reaction time for derivatization is relatively long, the procedure has a high derivatization yield and is essentially quantitative (Avgerinos and Hutt, 1987). Further purification, using solid phase extraction, is essential following derivatization to remove the excess (*R*)-NEA reagent and also serves as an addition clean-up step by removing a large portion of co-extracted endogenous material.

Typical chromatograms of an authentic standard, extracts of drug free serum and serum obtained from a volunteer following the administration of 400 mg of racemic ibuprofen are shown in Figure 2.3. No interfering peaks were observed in the chromatogram for the extract of drug free serum at the retention times of the analyte and internal standard derivatives. However, the chromatogram obtained following analysis of volunteer serum post drug administration revealed the presence of peaks, possibly corresponding to carboxyibuprofen, that co-eluted with the derivative of (*R*)-flurbiprofen and so quantitative analysis was performed using the (*S*)-flurbiprofen derivative peak as the internal standard. The values for extraction recoveries from serum were generally greater than 87 % (Tan *et al.*, 1997a).

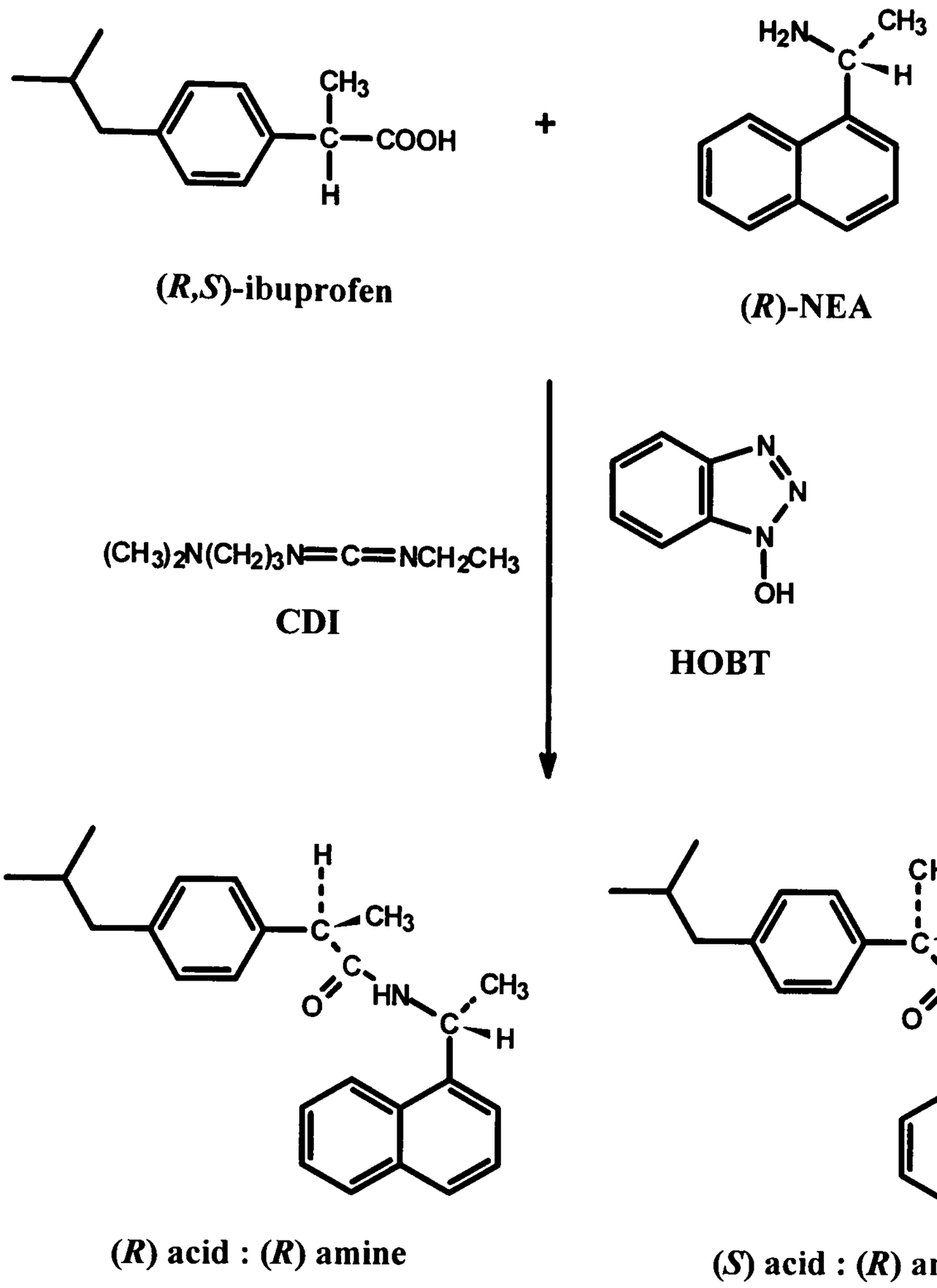


Figure 2.2: Derivatization of (*R*,*S*)-ibuprofen with (*R*)-1-(naphthen-1-yl)ethylamine ((*R*)-NEA) in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (CDI) and 1-hydroxybenzotriazole (HOBT).

Validation of assay procedure

The calibration curves for the analysis of ibuprofen enantiomers isolated from serum were linear between 0.1-10 μg/ml using fluorescence detection (excitation and emission wavelengths of 290 and 330 nm respectively) and are shown in Figure 2.4. Linear regression analysis of the calibration curves routinely gave correlation coefficients greater than 0.997.

Analytical precision and accuracy were established by adding known quantities of ibuprofen at three different concentrations to serum and analysing aliquots on a single day (n =6) and on six different assay days, and the results are presented in Table 2.1. The coefficient of variation of the analytical method for within- and between-day studies was generally less than 10 %, indicating the precision of the assay at all concentrations

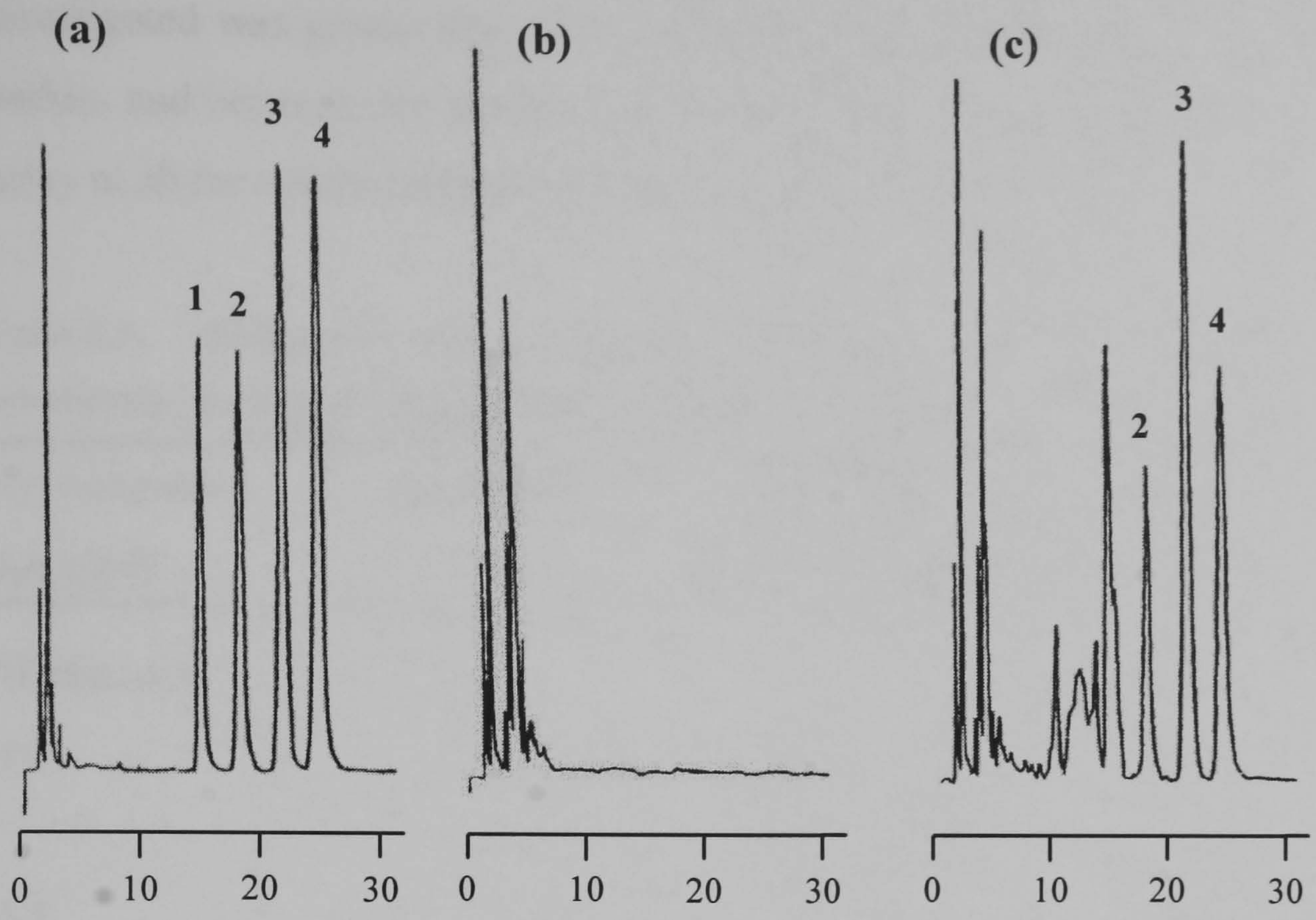


Figure 2.3: Chromatograms of (a) standard solution of (*R,S*)-ibuprofen derivatized with (*R*)-NEA, extracts of (b) blank serum and (c) a volunteer serum sample obtained 2hr post oral administration of the racemic drug. Peaks : (*R*)-1-(naphthen-1-yl)ethylamides of 1, (*R*)-flurbiprofen; 2, (*S*)-flurbiprofen (I.S.); 3, (*R*)-ibuprofen and 4, (*S*)-ibuprofen. [Mobile phase, phosphate buffer (pH 3.5, 0.01M):acetonitrile (50:50 v/v); Flow rate, 1.5 ml/min; Detection, fluorescence λex = 290 nm and λem = 330 nm].

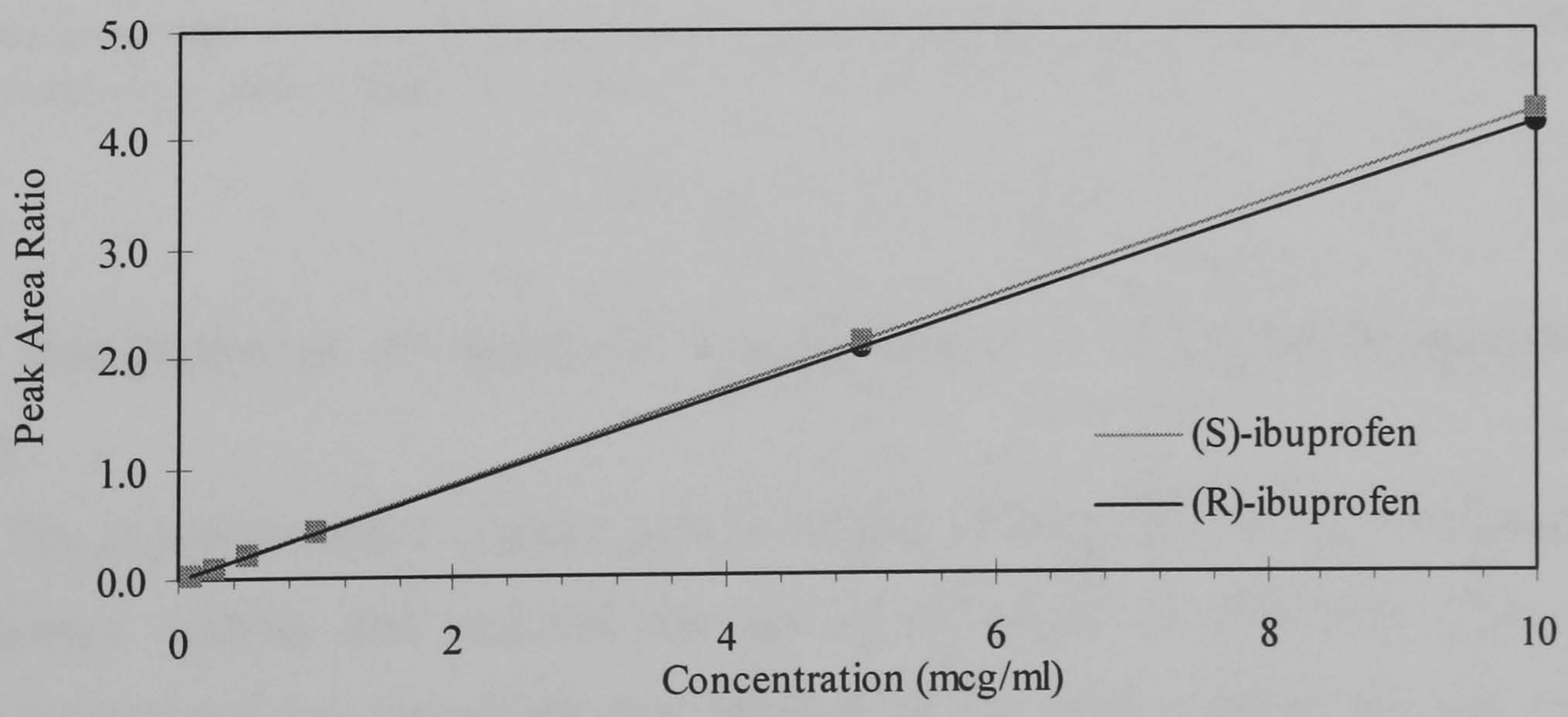


Figure 2.4: A typical calibration curve prepared for the quantification of the enantiomers of ibuprofen as their (*R*)-1-(naphthen-1-yl)ethylamides following extraction of the drug from serum.

investigated was greater than 90 %. The mean difference of the analytical method for within- and between-day studies was also less than 10 %, indicating the accuracy of the assay at all the concentrations examined was greater than 90 %.

Table 2.1: Within-day and between-day analytical variation and accuracy of ibuprofen enantiomers in "spiked" serum samples (mean ± s.d.; n=6) *.

Concentration (mcg/ml)	Enantiomer	Concentration determined (mcg/ml)	C.V. (%)	M.D. (%)
Within-day				
0.1	*R*	0.097 ± 0.005	5.63	-3.0
	S	0.098 ± 0.006	5.78	-2.0
1.0	*R*	1.05 ± 0.03	3.09	5.0
	S	1.07 ± 0.04	3.76	7.0
10.0	*R*	10.08 ± 0.26	2.56	0.8
	S	10.09 ± 0.28	2.81	0.9
Between-day				
0.1	*R*	0.095 ± 0.008	8.74	-5.0
	S	0.096 ± 0.007	7.48	-4.0
1.0	*R*	1.02 ± 0.04	4.40	2.0
	S	1.03 ± 0.06	5.64	3.0
10.0	*R*	10.01 ± 0.35	3.54	0.1
	S	10.04 ± 0.33	3.30	0.4

** C.V. = coefficient of variation; M.D. = mean difference.*

2.3.2 Determination of unbound concentrations of ibuprofen enantiomers in serum

The enantioselective plasma protein binding of ibuprofen was investigated using equilibrium dialysis and required the use of radiolabelled ibuprofen. The dialysis method employed was essentially that detailed by Tan *et al.* (1997a) and was based on an original method developed by Evans *et al.* (1989). Equilibrium dialysis was preferred over other methods of determining free concentrations since it is relatively cheap to perform and required the use of non-specialised instrumentation. The use of

radiolabelled drug is often necessitated as drugs which are highly protein bound, like the 2-arylpropionic acids, require free drug assays to be very sensitive as extremely low concentrations are encountered (Rowland, 1980: Wright *et al.*, 1996). Before utilisation in the dialysis experiments, the radiolabelled ibuprofen was purified by semi-preparative HPLC to remove low protein binding radiochemical impurities as their presence, even in small amounts, can lead to spurious estimates of unbound fractions (Bjornsson *et al.*, 1981). Based on the collection of timed fractions and radiochemical analysis, the purities pre- and post-chromatographic purification were about 96 % and 99 % respectively.

Separation of the ibuprofen enantiomers, following isolation from post-dialysis buffer and serum, was carried out using a direct chromatographic approach with a CSP, rather than using the indirect methods previously employed (Evans *et al.*, 1989; Tan *et al.*, 1997a). In this manner, higher throughput could be achieved and also loss of sample by additional manipulation could be minimised. The resolution was performed using a derivatized cellulose CSP, cellulose tris (3,5-dimethylphenylcarbamate) (Chiralcel OD), and under the experimental chromatographic conditions (*R*)- and (*S*)-ibuprofen had retention times of 12.5 and 15.9 minutes respectively with separation (α) and resolution factors (R_S) of 1.23 and 1.28 respectively. Chromatograms of an ibuprofen standard, and post-dialysis extracts of dialysate and serum are shown in Figure 2.5; the addition of carrier non-radiolabelled (*R,S*)-ibuprofen during sample preparation ensured visualisation of the ibuprofen peaks and minimised potential sorptive losses of the radiolabelled drug.

Volume shifts in equilibrium dialysis occur as a result of the movement of water from the recipient to the donor chamber causing dilution of the binding protein concentration and if uncorrected, will result in an overestimation of the unbound fraction (Lockwood and Wagner, 1983; Lima *et al.*, 1983). Correction for volume shift is especially significant if the unbound fraction of the drug is small (Huang, 1983). The volume shifts obtained for the dialysis experiments were typically less than 10 % and the unbound fractions calculated were routinely corrected for volume shift based on protein concentration determinations pre- and post- dialysis for the serum samples.

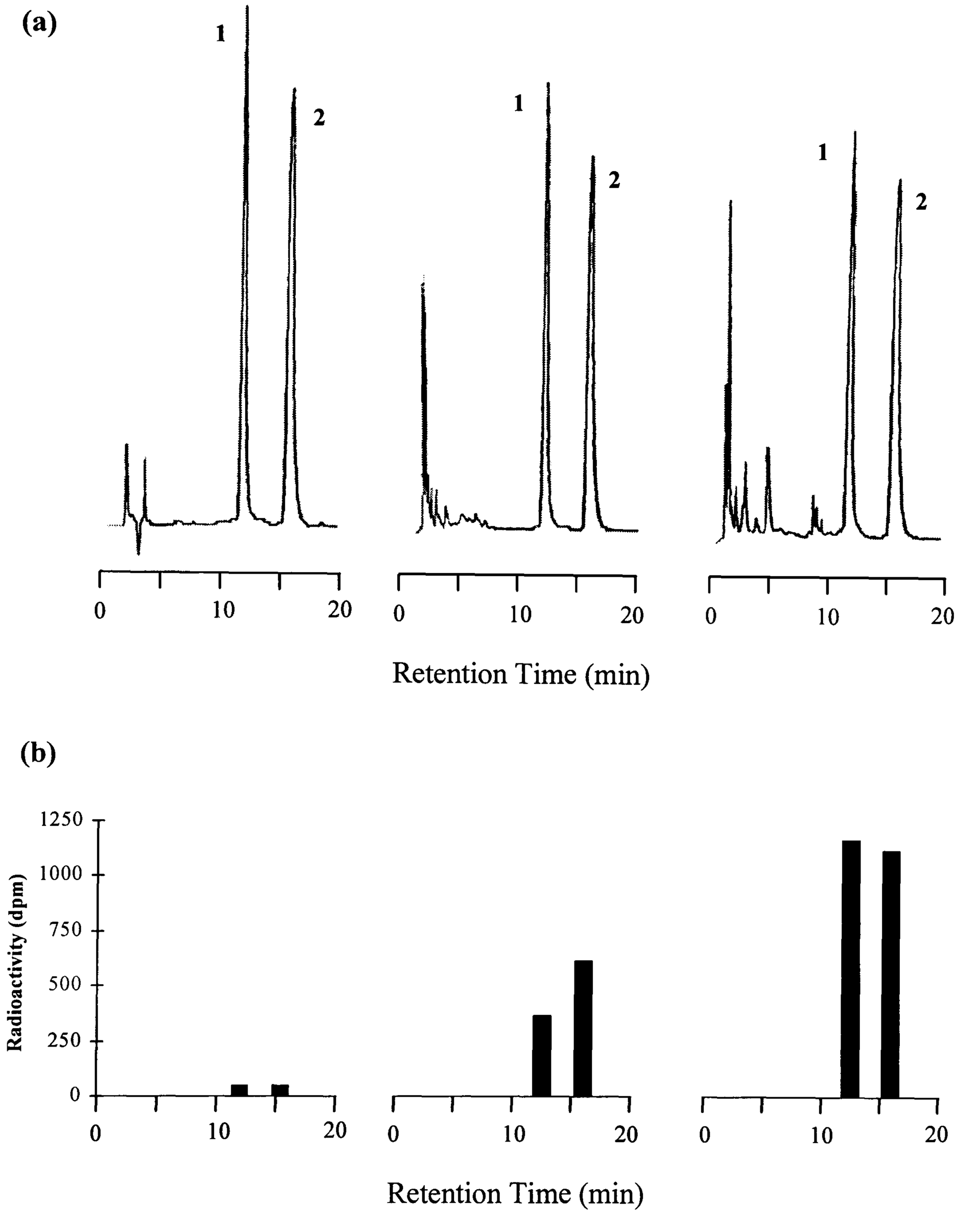


Figure 2.5: The (a) chiral-phase chromatograms and (b) radioactivity of (i) standard solutions of (*R,S*)-ibuprofen, post-dialysis extracts of (ii) buffer and (iii) "spiked" serum (10 μg/ml). Peaks : 1, (*R*)-ibuprofen; 2, (*S*)-ibuprofen [Mobile phase, hexane:isopropanol (100:1.1 v/v) with TFA (0.1 % v/v); Flow rate, 1.0ml/min; Detection, UV λ = 220 nm].

Validation

The reproducibility of the entire procedure for determining the unbound fractions of ibuprofen enantiomers was assessed at various analyte concentrations in serum and the results are presented in Table 2.2. In general the variation is greater than that seen for the serum assay validation experiments, this is not unexpected as much lower concentrations are involved and even a maximum coefficient of variation of 16 % is still within acceptable limits. Thus, although several steps are involved in the performance of this method, the overall reproducibility is acceptable.

Table 2.2: Precision data for the determination of the unbound fraction (%) for ibuprofen enantiomers (mean ± s.d.; n=5) *.

Enantiomeric Concentration (mcg/ml)	Enantiomer	Unbound fraction (%)	C.V. (%)
5	*R*	0.24 ± 0.023	9.6
	S	0.66 ± 0.021	3.2
10	*R*	0.25 ± 0.039	15.6
	S	0.68 ± 0.035	5.1
20	*R*	0.28 ± 0.043	15.4
	S	0.70 ± 0.069	9.9

** C.V. = coefficient of variation.*

These results also demonstrate that the protein binding of ibuprofen enantiomers to plasma protein is stereoselective, with the *R*-enantiomer binding to a greater extent than its *S*-antipode (Table 2.2). The extent of binding and degree of stereoselectivity was consistent with the observations of Evans *et al.* (1989). It is also worth noting that at the different concentrations assessed, which is expected to represent the typical therapeutic serum concentration range following the single oral administration of 400 mg ibuprofen (see Chapter 3), the enantiomeric binding was not significantly different and thus concentration-dependent protein binding is not anticipated to be an influential factor in the volunteer study.

2.3.3 Enantiospecific analysis of ibuprofen in urine

The method adopted to determine the concentrations of ibuprofen enantiomers in urine was essential identical to that employed for the serum assay (Tan *et al.*, 1997a). The only significant difference being that an alternative extraction solvent system, i.e. hexane:isopropanol (9:1, v/v), was used as diethylether was found to result in the co-extraction of large quantities of endogenous materials from urine. High extraction efficiencies was also achieved, with recovery values typically greater than 90 % for both enantiomers (Tan *et al.*, 1997a).

The determination of total (free plus conjugated) concentrations required the use of smaller aliquots of urine samples since liberation of ibuprofen from its conjugate was expected to result in much higher levels present in the samples. The hydrolysis of the ester glucuronides was performed using the base hydrolysis procedure optimised and utilised by Tan *et al.* (1997a). The use of alkaline rather than enzymatic hydrolysis of acyl glucuronides circumvents analytical problems associated with the intramolecular acyl migration of the carboxylic acid moiety to yield β-glucuronidase-resistant glucuronic acid esters (Faed, 1984; Caldwell *et al.*, 1983). Typical chromatograms of a standard, extracts of drug free urine and non-hydrolysed and hydrolysed volunteer urine samples are shown in Figure 2.6 and illustrate the absence of interfering peaks at the retention times of the derivatives of (*R*)- and (*S*)-ibuprofen and (*S*)-flurbiprofen, the internal standard.

Validation of the assay procedure

Single enantiomer calibration curves for ibuprofen (range: 0.1-10 μg/ml) typically yielded correlation coefficients greater than 0.997 and are shown in Figure 2.7. The within-day and between-day precision and accuracy values for the enantiomers of ibuprofen, as presented in Table 2.3, are generally good with coefficients of variation and mean differences less than 9 % at the different concentrations examined. These values were comparable to those obtained for the serum assay.

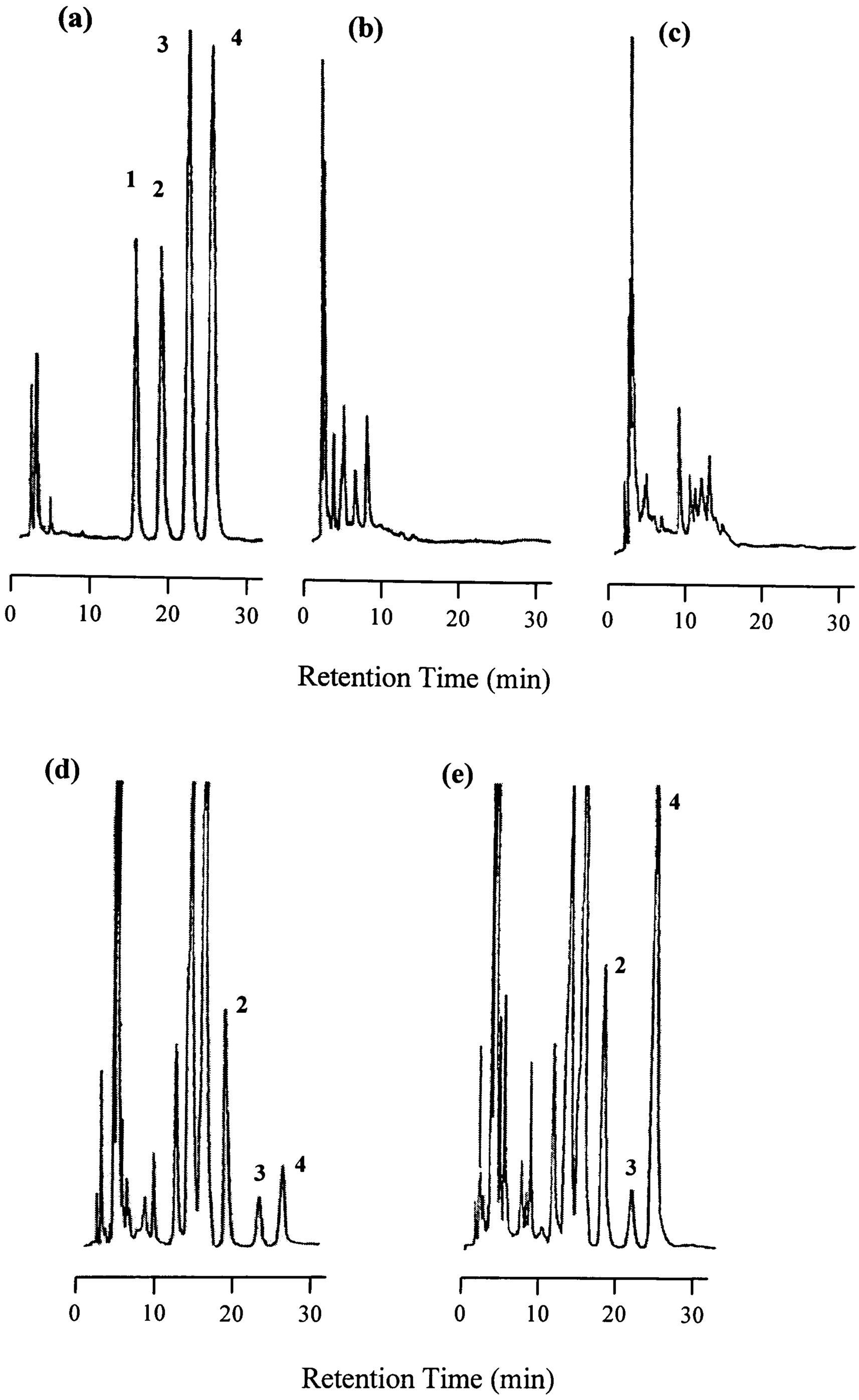


Figure 2.6: Chromatograms of (a) standard solution of (*R*,*S*)-ibuprofen derivatized with (*R*)-NEA, extracts of (b) non-hydrolysed and (c) hydrolysed blank urine and (d) non-hydrolysed and (e) hydrolysed volunteer 24 hour pooled urine samples following oral administration of the racemic drug. Peaks, (*R*)-1-(naphthen-1-yl)ethylamide derivatives of : 1, (*R*)-flurbiprofen; 2, (*S*)-flurbiprofen (I.S.); 3, (*R*)-ibuprofen and 4, (*S*)-ibuprofen [Mobile phase, phosphate buffer (pH 3.5, 0.01M):acetonitrile (50:50, v/v); Flow rate, 1.5 ml/min; Detection, fluorescence λex = 290 nm and λem = 330 nm].

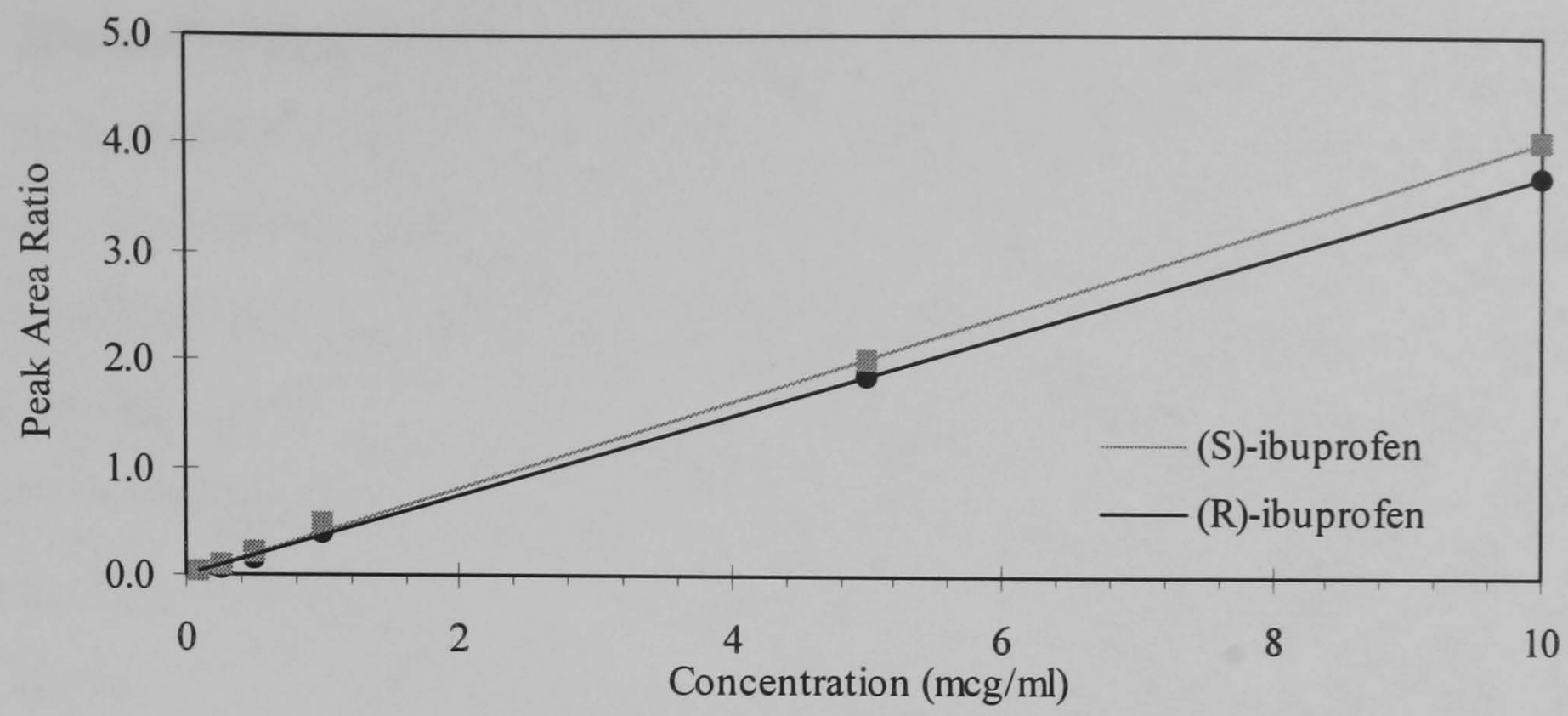


Figure 2.7: A typical calibration curve prepared for the quantification of the enantiomers of ibuprofen as their (*R*)-1-(naphthen-1-yl)ethylamides following extraction of the drug from urine.

Table 2.3: Within-day and between-day analytical variation and accuracy of ibuprofen enantiomers in "spiked" urine samples (mean ± s.d.; n=6) *.

Concentration (mcg/ml)	Enantiomer	Concentration determined (mcg/ml)	C.V. (%)	M.D. (%)
Within-day				
0.1	*R*	0.094 ± 0.005	5.36	-6.0
	S	0.096 ± 0.006	6.08	-4.0
1.0	*R*	0.99 ± 0.04	3.57	-1.0
	S	1.00 ± 0.03	2.96	0.0
10.0	*R*	10.01 ± 0.29	2.86	0.1
	S	10.03 ± 0.25	2.45	0.3
Between-day				
0.1	*R*	0.094 ± 0.008	8.31	-6.0
	S	0.097 ± 0.007	6.99	-3.0
1.0	*R*	0.99 ± 0.04	4.34	-1.0
	S	0.98 ± 0.03	3.14	-2.0
10.0	*R*	10.05 ± 0.32	3.21	0.5
	S	10.06 ± 0.30	2.97	0.6

** C.V. = coefficient of variation; M.D. = mean difference.*

2.3.4 Stereospecific analysis of carboxyibuprofen and hydroxyibuprofen in urine

A sequential achiral-chiral HPLC method was adopted for the stereospecific analysis of carboxyibuprofen and hydroxyibuprofen in urine; the metabolite concentrations are determined on the achiral-phase and the appropriate fraction of the eluate transferred to a CSP for the determination of stereochemical composition (Tan *et al.*, 1997b). Achiral chromatography is used as a first-step separation to obtain good resolution between the structurally similar metabolites and to minimise the exposure of the CSP to co-extracted contaminants which could potentially effect its stability or modify its separation properties (Ducharme *et al.*, 1996; Hutt and Patel, 1998).

Achiral chromatography was performed using a silica stationary phase, as the normal-phase conditions are compatible with those of the CSP and facilitate sample manipulation in that the collected eluate can be readily evaporated to concentrate the analyte before introduction onto the CSP. Typical chromatograms of a standard, extracts of drug free urine and non-hydrolysed and hydrolysed volunteer urine samples are shown in Figure 2.8. No interfering peaks were observed in the chromatograms at the retention times of either metabolite or the internal standard owing to endogenous constituents of urine either before or after alkali treatment to hydrolyse the ester glucuronide conjugates. Extraction recoveries were generally greater than 90 % and 93 % for carboxyibuprofen and hydroxyibuprofen respectively (Tan *et al.*, 1997b).

Chiral chromatography was carried out using a derivatized amylose CSP, amylose tris (3,5-dimethylphenylcarbamate) (Chiralpak AD), under chromatographic conditions used previously for the resolution and characterisation of the stereoisomers of carboxyibuprofen (Tan *et al.*, 1997c). Chromatograms of a carboxyibuprofen standard and of eluate fractions collected following achiral analysis of extracts of drug free urine and non-hydrolysed and hydrolysed volunteer urine samples are shown in Figure 2.9. The same mobile phase conditions were also used for the analysis of hydroxyibuprofen and typical chromatograms are shown in Figure 2.10. As would be expected the chiral-phase chromatograms of the metabolites, following their separate collection using normal-phase chromatography, were extremely clean with the chromatograms for the "blanks" showing stable baselines after the initial solvent fronts.

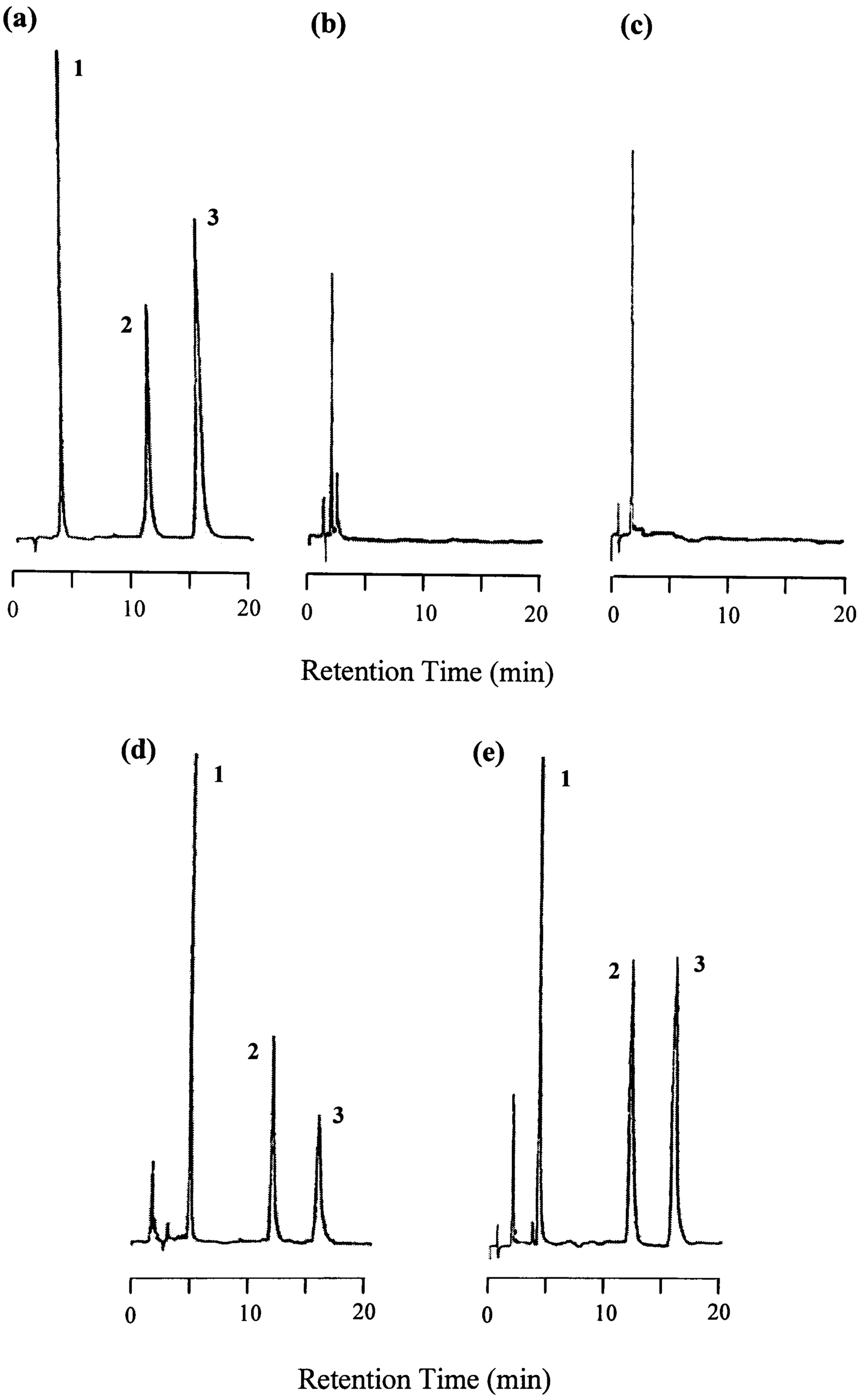


Figure 2.8: Normal-phase chromatograms of (a) reference standards, extracts of (b) non-hydrolysed and (c) hydrolysed blank urine and (d) non-hydrolysed and (e) hydrolysed volunteer 24 hour pooled urine samples following oral administration of the racemic drug. Peaks : 1, *p*-chloro-phenoxyacetic acid (I.S.); 2, carboxyibuprofen and 3, hydroxyibuprofen [Mobile phase, hexane:ethanol (98.2:1.8 v/v) with TFA (0.05% v/v); Flow rate, 2.0 ml/min; Detection, UV λ = 220 nm].

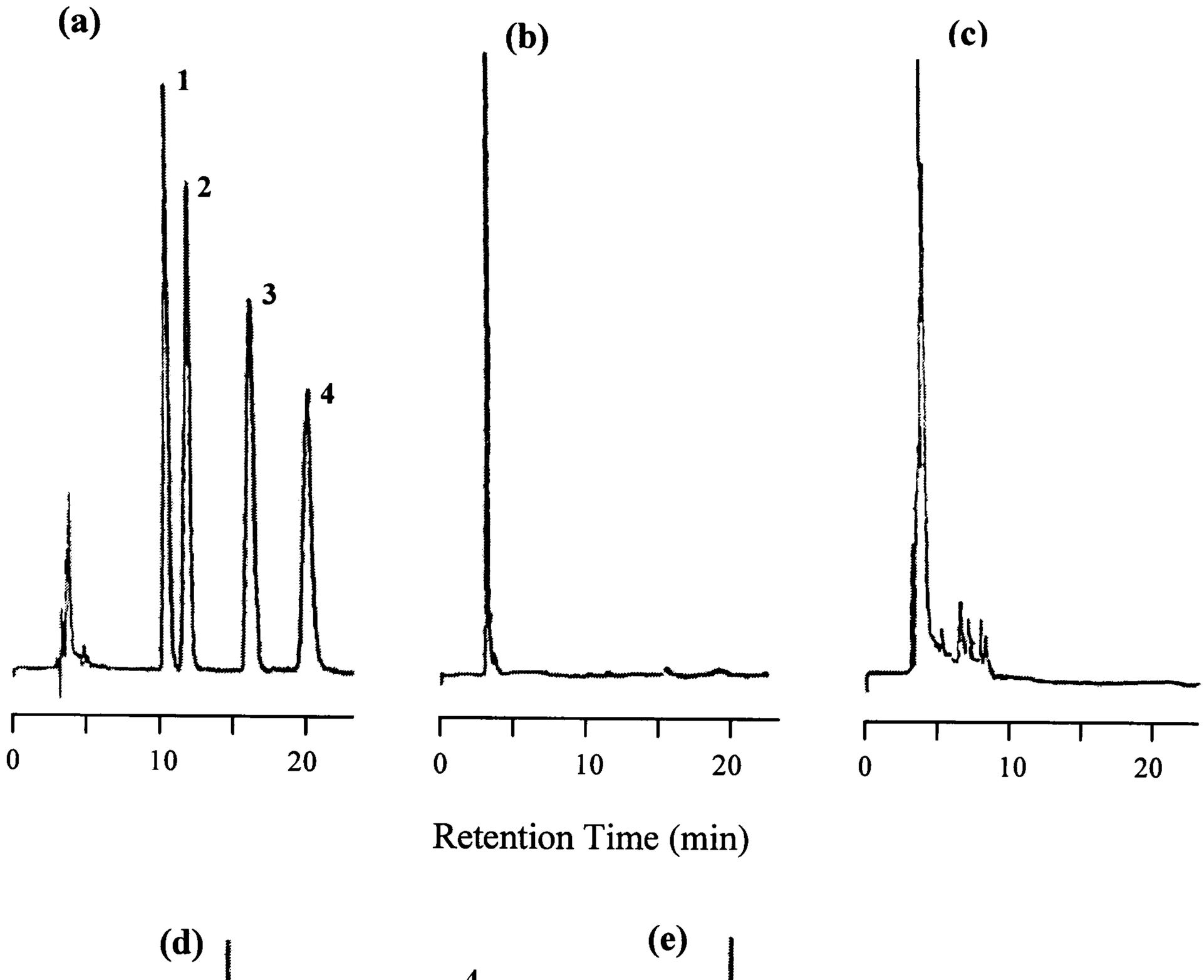
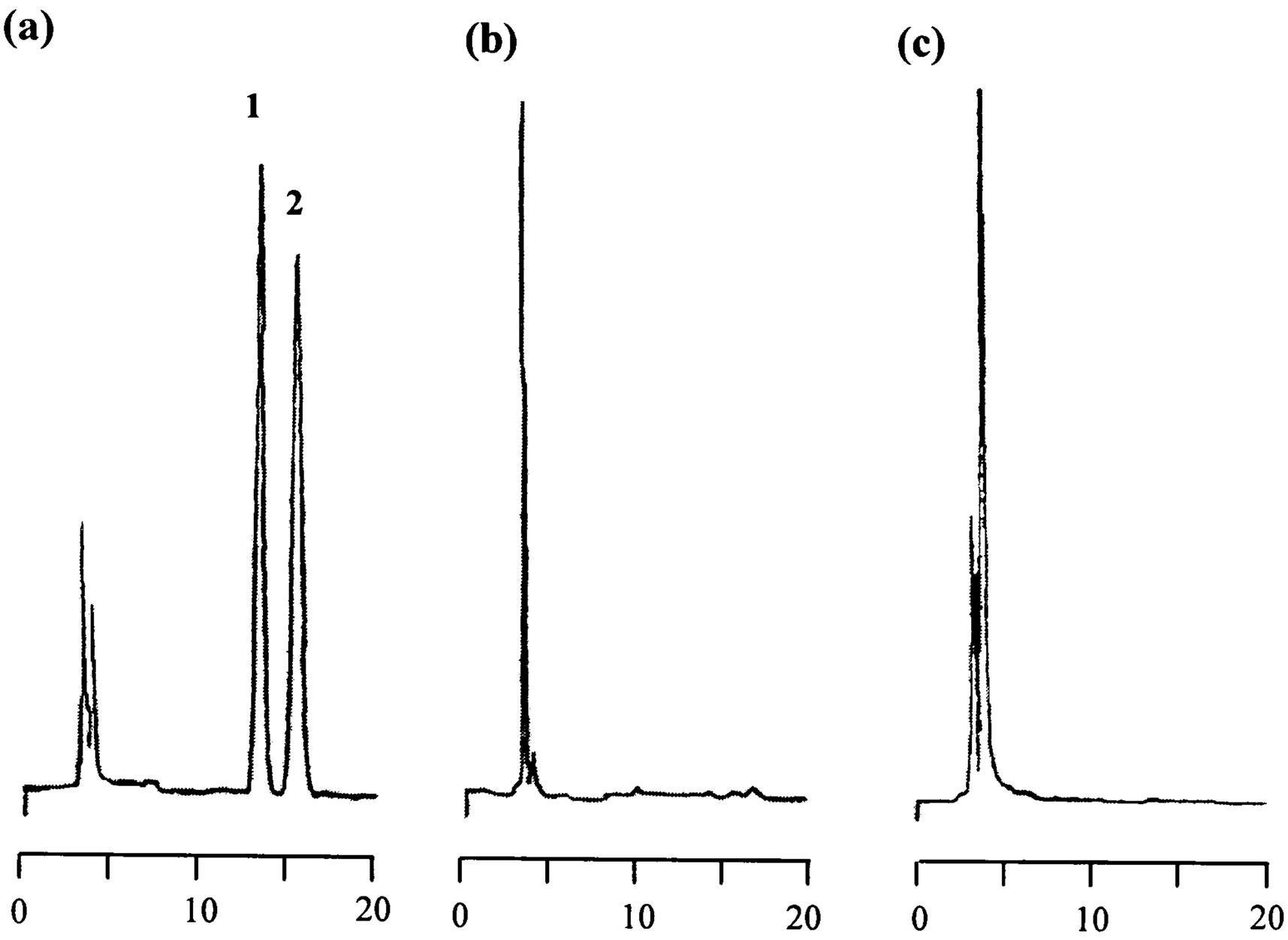


Figure 2.9: Chiral-phase chromatograms of (a) carboxyibuprofen standard, normal-phase HPLC eluate collections from (b) non-hydrolysed and (c) hydrolysed blank urine and (d) non-hydrolysed and (e) hydrolysed volunteer 24 hour pooled urine samples following oral administration of the racemic drug. Peaks : 1, (2'*S*,2*R*)-; 2, (2'*R*,2*R*)-; 3, (2'*R*,2*S*)- and 4, (2'*S*,2*S*)- [Mobile phase, hexane:ethanol (92:8 v/v) with TFA (0.05% v/v); Flow rate, 1.0 ml/min; Detection, UV λ = 220 nm].



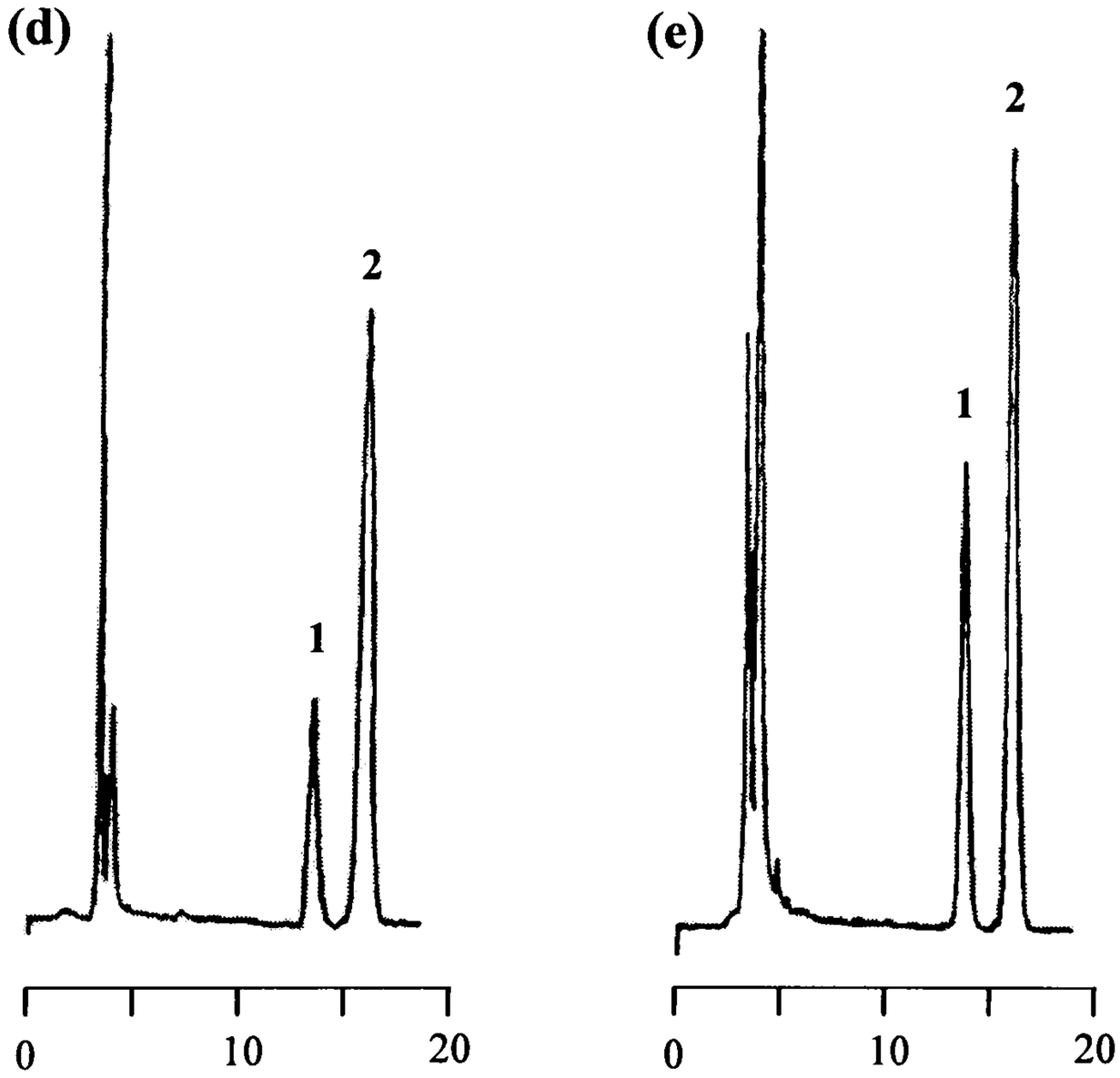


Figure 2.10: Chiral-phase chromatograms of (a) hydroxyibuprofen standard, normal-phase HPLC eluate collections from (b) non-hydrolysed and (c) hydrolysed blank urine and (d) non-hydrolysed and (e) hydrolysed volunteer 24 hour pooled urine samples following oral administration of the racemic drug. Peaks : 1, (*R*)-hydroxyibuprofen and 2, (*S*)-hydroxyibuprofen [Mobile phase, hexane:ethanol (92:8 v/v) with TFA (0.05% v/v); Flow rate, 1.0 ml/min; Detection, UV λ = 220 nm].

Validation of the assay procedure

The calibration curves for the quantification of carboxy and hydroxyibuprofen extracted from urine were linear in the range of 10-320 μg/ml and are shown in Figure 2.11. Linear regression analysis of the data produced correlation coefficients greater than 0.997 for both metabolites. The achiral-phase analytical procedure showed acceptable precision, accuracy and between-day variability for both analytes at the three concentration levels examined, with coefficients of variation and mean differences less than 8 % and 3 % respectively (Tables 2.4 and 2.5).

The chiral-phase method was validated by analysing the material in the eluate collected from the above normal-phase validation experiments. These further validation studies are fundamental to determine whether the chiral assay is able to reproduce the expected stereochemical compositions throughout the complete analytical procedure and over a wide range of analyte concentrations. The precision and accuracy values of the calculated stereochemical compositions for both metabolites are presented in Tables 2.6 and 2.7. The results indicate that there was little variation from the expected stereochemical compositions over a wide range of concentrations, which suggests that metabolite concentration does not result in chiral discrimination using the sequential chromatographic approach. Thus, the measurement of the stereochemical composition of the metabolites in the eluate from the normal-phase system can be determined with high degrees of accuracy and precision using the Chiralpak AD CSP.

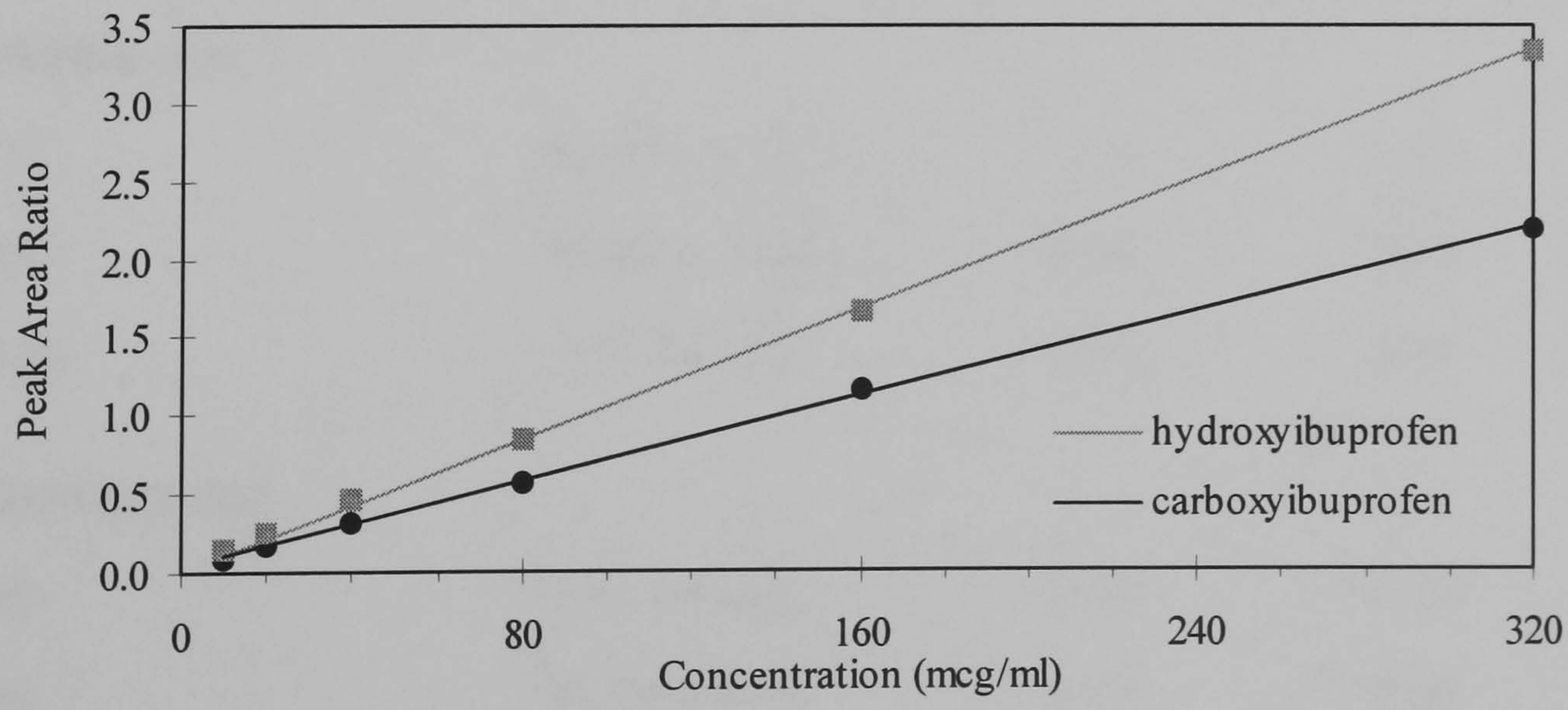


Figure 2.11: Typical calibration curves prepared for the quantification of carboxyibuprofen and hydroxyibuprofen using the normal-phase HPLC assay.

Table 2.4: Within-day and between-day analytical variation and accuracy of carboxyibuprofen in "spiked" urine samples (mean ± s.d.; n=6) *.

Concentration (mcg/ml)	Concentration determined (mcg/ml)	C.V. (%)	M.D. (%)
Within-day			
10	9.96 ± 0.416	4.18	-0.40
80	80.45 ± 2.27	2.82	0.56
320	322.3 ± 9.2	2.84	0.72
Between-day			
10	10.11 ± 0.616	6.10	1.10
80	81.68 ± 4.27	5.22	2.33
320	323.7 ± 12.1	3.75	1.16

* *C.V. = coefficient of variation; M.D. = mean difference.*

Table 2.5: Within-day and between-day analytical variation and accuracy of hydroxyibuprofen in "spiked" urine samples (mean ± s.d.; n=6) *.

Concentration (mcg/ml)	Concentration determined (mcg/ml)	C.V. (%)	M.D. (%)
Within-day			
10	10.19 ± 0.545	5.35	1.90
80	80.53 ± 3.29	4.08	0.66
320	322.9 ± 9.9	3.05	0.91
Between-day			
10	9.93 ± 0.641	6.46	-0.70
80	81.74 ± 4.19	5.13	2.18
320	318.1 ± 25.2	7.93	-0.59

* *C.V. = coefficient of variation; M.D. = mean difference.*

Table 2.6: Within-day and between-day analytical variation and accuracy of stereochemical compositions determined by the analysis of eluate from normal-phase chromatography of urine samples "spiked" with carboxyibuprofen (mean ± s.d.; n=6) *.

"Total" concentration (mcg/ml)	Isomer	Stereochemical composition (%)	C.V. (%)	M.D. (%)
Within-day				
10	*2'S,2R*	25.01 ± 1.11	4.44	0.04
	2'R,2R	25.08 ± 1.52	6.06	0.32
	2'R,2S	25.03 ± 1.35	5.39	0.12
	2'S,2S	24.89 ± 1.94	7.79	-0.44
80	*2'S,2R*	25.04 ± 0.84	3.36	0.16
	2'R,2R	25.04 ± 1.12	4.47	0.16
	2'R,2S	24.98 ± 1.43	5.72	-0.08
	2'S,2S	24.94 ± 1.66	6.66	-0.24
320	*2'S,2R*	25.03 ± 1.21	4.83	0.12
	2'R,2R	25.01 ± 0.74	2.96	0.04
	2'R,2S	24.98 ± 1.10	4.40	-0.08
	2'S,2S	24.99 ± 1.84	7.36	-0.04
Between-day				
10	*2'S,2R*	24.99 ± 1.44	5.76	-0.04
	2'R,2R	25.02 ± 1.65	6.59	0.08
	2'R,2S	25.02 ± 1.54	6.16	0.08
	2'S,2S	24.98 ± 1.98	7.93	-0.08
80	*2'S,2R*	25.05 ± 0.33	1.32	0.20
	2'R,2R	24.97 ± 1.61	6.45	-0.12
	2'R,2S	25.04 ± 1.09	4.35	0.16
	2'S,2S	24.93 ± 1.86	7.46	-0.28
320	*2'S,2R*	25.08 ± 0.73	2.91	0.32
	2'R,2R	25.03 ± 1.03	4.12	0.12
	2'R,2S	24.94 ± 1.23	4.93	-0.24
	2'S,2S	24.95 ± 0.92	3.69	-0.20

** C.V. = coefficient of variation; M.D. = mean difference.*

Table 2.7: Within-day and between-day analytical variation and accuracy of enantiomeric compositions determined by the analysis of eluate from normal-phase chromatography of urine samples "spiked" with hydroxyibuprofen (mean ± s.d.; n=6) *.

"Total" concentration (mcg/ml)	Enantiomer	Enantiomeric composition (%)	C.V. (%)	M.D. (%)
Within-day				
10	*R*	50.02 ± 1.72	3.44	0.04
	S	49.98 ± 1.73	3.46	-0.04
80	*R*	50.04 ± 1.23	2.46	0.08
	S	49.96 ± 1.23	2.46	-0.08
320	*R*	50.00 ± 0.93	1.86	0.00
	S	50.00 ± 0.93	1.86	0.00
Between-day				
10	*R*	50.11 ± 2.82	5.62	0.22
	S	49.89 ± 2.81	5.63	-0.22
80	*R*	50.01 ± 1.32	2.64	0.02
	S	49.99 ± 1.32	2.64	-0.02
320	*R*	50.07 ± 1.19	2.38	0.14
	S	49.93 ± 1.18	2.36	-0.14

** C.V. = coefficient of variation; M.D. = mean difference.*

2.4. Summary

In summary, this chapter has described HPLC methods for the stereospecific analysis of ibuprofen and its major metabolites in biological fluids. Enantiospecific analysis of ibuprofen in serum and urine was based on the indirect approach to chiral chromatography, involving derivatization with (*R*)-1-(naphthen-1-yl)ethylamine to yield fluorescent diastereomeric amides which can be resolved using reversed-phase HPLC. Free, non-protein bound, ibuprofen was determined in serum by equilibrium dialysis using radiolabelled ibuprofen and the direct resolution of the enantiomers on a derivatized cellulose CSP. Determination of the stereochemical composition of carboxy and hydroxyibuprofen in urine was performed by sequential achiral-chiral chromatography, the metabolites were separated and quantified using a normal-phase

HPLC system and the respective eluates collected for determination of stereochemical composition using a derivatized amylose CSP. Validation experiments were performed for all the assays and showed that they were suitable for utilisation in quantitative analysis. The application of the analytical methodologies to human pharmacokinetic studies following the oral administration of racemic ibuprofen to healthy young and elderly volunteers is presented in the following Chapter.

CHAPTER 3 :

Stereoselective disposition and pharmacodynamics of ibuprofen in young and elderly volunteers

3.1. Introduction

Clinical investigations into the disposition of ibuprofen enantiomers need to take into consideration three major aspects; namely, chiral inversion, metabolite formation and protein binding. As ibuprofen is predominately used as the racemate, the unidirectional chiral inversion of the relatively inactive *R*-enantiomer to the active *S*-antipode is essentially a form of metabolic activation, the extent of which needs to be investigated to estimate the effective dose of the active agent. Variability in chiral inversion will also influence other stereoselective transformations and may cause significant differences in enantiomeric disposition (Caldwell *et al.*, 1988a).

In addition to chiral inversion, ibuprofen undergoes functional oxidative and conjugative metabolism. The principal metabolic pathway is oxidation of the isobutyl side-chain to yield hydroxyibuprofen and carboxyibuprofen. The formation of the carboxy metabolite introduces a second chiral centre into the molecule and due to the chromatographic resolution problems outlined in the previous chapter it has been difficult to discern the substrate or product enantioselectivity of the reaction. Excretion of ibuprofen as the acyl glucuronide is quantitatively a relatively minor metabolic pathway, accounting for only 10 % of the dose. However, *in vitro* experiments have revealed glucuronidation to be stereoselective, favouring (*S*)-ibuprofen as the substrate (El Mouelhi *et al.*, 1987).

Ibuprofen, in common with most NSAIDs, is extensively bound to plasma proteins with unbound fractions of typically less than 0.01 (Gallo *et al.*, 1986; Lin *et al.*, 1987). *In vitro* and *in vivo* studies have also shown the plasma protein binding to be stereoselective, with the *S*-enantiomer having a significantly higher unbound fraction (Evans *et al.* 1989; Lapicque *et al.*, 1993; Paliwal *et al.*, 1993; Hage *et al.*, 1995). Furthermore, each ibuprofen enantiomer has been shown to display non-linear protein binding and over the range of concentrations found clinically, was able to displace its optical antipode from protein binding sites (Evans *et al.*, 1990; Paliwal *et al.*, 1993). In terms of pharmacokinetics, the protein binding will have an influence on distribution and elimination. The enantiomers of ibuprofen would be expected to have relatively small volumes of distribution since their strong association with plasma proteins confines them to vascular spaces (Lin *et al.*, 1987). For a low clearance drug like ibuprofen, the rate of hepatic metabolism is low, such that binding is a limiting factor in clearance. The clearance of each enantiomer of ibuprofen is, therefore, directly

proportional to their respective free fractions in blood (Lin *et al.*, 1987). Thus, pharmacokinetic studies based on unbound concentrations are essential to yield information on the enantioselectivity of the underlying dispositional processes. However, such studies are often not performed due to the difficulties associated with developing methods capable of detecting very low unbound concentrations.

The first study on the pharmacokinetics of ibuprofen enantiomers in man reported a difference in plasma elimination half lives for the *R*- and *S*-enantiomers and the predominance of (*S*)-ibuprofen in urine (Van Giessen and Kaiser, 1975). Since then, the considerable interest in the stereochemical aspects of its disposition, together with the possible implications of the chiral inversion reaction has lead to the publication of numerous other enantioselective dispositional studies of ibuprofen. A recent review article concerned with the clinical pharmacokinetics of ibuprofen cited no less than 40 different stereospecific studies (Davies, 1998). However, the vast majority of these studies are not comprehensive and only addressed some of the important issues outlined above. Furthermore, very little attention has been paid to studying the effect of ageing on the enantioselectivity of ibuprofen disposition, which is rather surprising considering the use of NSAIDs by the elderly is three fold that of the young (Baum *et al.*, 1985).

In general, studies of the influence of age on the pharmacokinetics and disposition of ibuprofen are scarce and have been predominantly based on non-stereospecific analysis. In the study of Albert *et al.* (1984), the pharmacokinetics of ibuprofen were evaluated after single oral doses of 400, 800 and 1200 mg to 17 elderly volunteers (age range: 65-78 years) and the data obtained compared with parameters determined from a previous study involving 15 young healthy male volunteers (age range: 22-35 years). No age-related differences were observed for total and unbound pharmacokinetic parameters suggesting that no dose adjustment is necessary in the elderly. Another study by Greenblatt *et al.* (1984) investigated the pharmacokinetics of a single 600 mg dose of ibuprofen in healthy elderly (age range: 60-88 years) and young (age range: 22-44 years) volunteers and the results obtained gave a conflicting picture. Ibuprofen elimination half-life was longer and clearance reduced in elderly males compared with young male subjects. A more recent pharmacokinetic comparison of ibuprofen involving the administration of a sustained release tablet formulation, to younger (age range: 19-60 years) and elderly (age range: 65-85 years) patients for a two week period demonstrated comparable pharmacokinetics without appreciable drug accumulation (Kendall *et al.*, 1990).

The main limitation of the above age-related pharmacokinetic studies is that the enantiomeric composition of ibuprofen in plasma has not been taken into consideration. As ageing can affects various pharmacokinetic parameters which exhibit stereo-selectivity, e.g. protein binding, hepatic metabolism and renal elimination, it is thus conceivable that the disposition of ibuprofen may display age-related stereoselective differences in these mechanisms. A relatively recent investigation of the enantiomeric disposition of racemic ibuprofen in healthy young volunteers (age range: 24-34 years), healthy elderly volunteers (age range: 65-80 years) and elderly patients with renal impairment (age range: 66-87 years) showed a stereoselective increase in the unbound fraction of (*S*)-ibuprofen in both elderly groups (Rudy *et al.*, 1995). In addition, the pharmacokinetics of (*S*)-ibuprofen were significantly different in the renally imparied group compared to the young, the elimination half-life was increased and unbound clearance decreased.

Furthermore, no studies are present in the literature which have investigated the effects of age on pharmacodynamic responses to ibuprofen. Pharmacodynamic activity has been shown to relate closely to plasma concentrations of unbound (*S*)-ibuprofen rather than total levels since only unbound drug is available for pharmacological interactions with enzymes (Lin *et al.*, 1987). Since age associated differences in (*S*)-ibuprofen plasma protein binding has been reported (Rudy *et al.*, 1995), it is possible that pharmacodynamic responses may differ between the young and elderly. As outlined in Chapter One, the primary pharmacological activity of (*S*)-ibuprofen is to decrease the bio-synthesis of prostaglandins and thromboxanes by inhibiting the enzyme cyclooxygenase. In platelets, cyclooxygenase activity is stimulated following platelet activation which occurs in response to a variety of physiological processes including haemostasis and inflammation. The activity of ibuprofen *in vivo* can therefore be determined by monitoring end-points of the enzyme's activity, i.e. by following biochemical (e.g. thromboxane B_2) and functional (e.g. platelet aggregation) markers (Evans *et al.*, 1991).

The main objective of the current investigation is to examine the disposition of ibuprofen enantiomers following the oral administration of the racemic drug (400 mg) to healthy young and elderly volunteers; important issues that will be addressed include the extent and variability of chiral inversion, stereoselectivity of the major metabolic pathways as well as stereoselectivity in protein binding. In addition, the effect of age on the pharmacodynamic activity of ibuprofen, i.e. inhibition of cyclooxygenase, will be

investigated by determining *ex vivo* thromboxane concentrations in clotting blood and monitoring platelet aggregability.

3.2. Experimental

3.2.1 Clinical study protocol

Volunteers

Two groups of subjects were studied : a) eight young healthy volunteers aged from 20 to 31 years (mean ± s.d., 24.1 ± 3.7 years), four male and four female; and b) eight elderly healthy volunteers aged from 66 to 84 years (75.6 ± 6.8 years), seven male and one female; their demographic data is shown in Appendix 1.Volunteers were judged to be in good health on the basis of case history, physical examination, ECG, and routine laboratory data including blood pressure and pulse. The study was approved by the Research Ethics Committee of the School of Medicine and Dentistry, King's College London. Volunteers were fully informed about the investigational procedure and the use of ibuprofen. In compliance with the Declaration of Helsinki, written informed consent was obtained from each participant before enrolment in the study.

Study design

The volunteers were required to abstain from any alcohol, caffeine-based products and medication for at least 24 hours prior to the study. The volunteers were fasted for at least 8 hours overnight before a single 400mg oral dose of racemic ibuprofen (Brufen®) was administered with 150ml of water. Food and drink were withheld for at least 3 hours after drug administration.

Blood samples were collected immediately before drug administration and at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 3.5, 4.0, 6.0, 8.0, 10 and 24 hours post dosing in plain silicone coated vacutainer tubes. The volume of blood collected was 10 ml except at 0, 2, 4 and 8 hours where an additional 10 ml of blood was required for use in platelet aggregation studies, 1.5 ml aliquots of all the samples collected were reserved for determination of thromboxane concentrations. The venous blood samples were drawn for the initial 10 hour period via an indwelling cannula, to avoid repeated venipuncture.

placed in a convenient vein in the arm of the volunteer. The cannula was flushed after each sample collection with 0.9% NaCl solution B.P. to avoid clotting in the cannula. Samples were allowed to clot for two hours and serum obtained after centrifugation (10 min at 2000 g). Urine samples were collected every two hours for the first 10 hours and then at 24 hours post drug administration. The individual urine volumes were recorded and a pooled 24 hour sample was prepared from the individual collections. Serum and urine samples were frozen at -20°C until required for analysis.

3.2.2 Serum and urine analysis

(*R*)- and (*S*)-Ibuprofen concentrations in serum and pooled urine were determined by the indirect chromatographic methods described in sections 2.2.4 and 2.2.6 respectively. The enantiomeric and diastereomeric composition of hydroxy- and carboxyibuprofen in pooled urine were determined using the achiral-chiral method detailed in section 2.2.7. All samples were analysed in duplicate and a set of appropriate calibration standards analysed on each day.

3.2.3 Serum protein binding

The fraction unbound for the enantiomers of ibuprofen was determined for five replicates using the equilibrium dialysis methodology described in section 2.2.5, following the pooling of serum samples obtained at 0.5, 1.0 2.0, 3.0 and 4.0 hours post-administration for each volunteer. The mean values for unbound fractions of (*R*)- and (*S*)-ibuprofen determined from these five replicate were used to calculate unbound enantiomeric serum concentrations.

3.2.4 Pharmacodynamic Studies

Thromboxane concentrations in serum

Thromboxane B_2 (TXB_2) concentrations in blood samples were determined using a validated radioimmunoassay by Dr Grazyna Kinkowska of the Department of Pharmacology, King's College London. Briefly, blood samples (250 µl; n = 4) taken prior to and at each collection point post-dosing were allowed to clot at 37°C for one

hour and TXB_2 extracted by mixing with ethanol (250 µl), formic acid (1 M; 250 µl) and ethyl acetate (1.75 ml). Following centrifugation, an aliquot of ethyl acetate (25 µl) was evaporated and TXB_2 concentrations determined using a TXB_2-specific antibody and [^{3}H]-TXB_2 by β-scintillation counting. Calibration curves were generated using standard solutions of TXB_2.

Platelet aggregation

Ex vivo platelet aggregation studies were performed by Dr Linda Chirrey of the Clinical Age Research Unit (CARU), School of Medicine and Dentistry, King's College London, for blood samples taken pre and 2, 4 and 8 hours post drug administration. Briefly, aliquots of freshly generated platelet rich plasma (250 µl) in the presence of a range of adenosine diphosphate (ADP) concentrations (25 µl of 0, 1, 2, 5 and 10 µM) were assessed using a platelet aggregometer. Platelet aggregation data was expressed as percentages of theoretical maximum at each collection time point.

3.2.5 Pharmacokinetic and data analysis

Determination of pharmacokinetic parameters was performed by conventional non-compartmental analysis of the enantiomeric serum concentration-time profiles of ibuprofen (Rowland and Tozer, 1995).

Maximum serum concentrations (C_{max}) and the corresponding times (T_{max}) were determined by examination of the individual serum concentration-time curves for each volunteer. The terminal elimination half-life ($t_{1/2,z}$) was determined using :

$$\mathbf{t_{1/2,z} = \ln 2/\lambda_z} \qquad \text{(Eqn. 3.1)}$$

where the elimination rate constant (λ_z) is obtained by log-linear regression of the terminal phase of the serum concentration-time profile. The area under the curve (AUC) was calculated by :

$$\mathbf{AUC = AUC_{0\rightarrow t} + AUC_{t\rightarrow\infty}} \qquad \text{(Eqn. 3.2)}$$

where AUC upto the last measurable data point ($AUC_{0\rightarrow t}$) was determined using the trapezoidal rule and extrapolated to infinite time ($AUC_{t\rightarrow\infty}$) by dividing the last

measurable serum concentration by λ_z. The area extrapolated beyond the last data point typically constituted less than 2 % of the total area.

The fraction of (*R*)-ibuprofen undergoing inversion (F_{inv}) was calculated from an examination of the stereochemical composition of the drug and both major metabolites (free and conjugated) excreted in urine over the 24 hour collection period (Rudy *et al.*, 1991):

$$F_{inv} = \frac{\%\ S \text{ metabolites} - \%\ S \text{ administered dose}}{\%\ R \text{ administered dose}} \qquad \text{(Eqn. 3.3)}$$

where "% *S* administered dose" and the "% *R* administered dose" are the percent of the administered dose with the *S* and *R* configuration respectively, i.e.50 % in each case; and "% *S* metabolites" is a summation of the molar amounts of metabolites excreted in urine with the *S* configuration in the propionic acid moiety, expressed as a percent of that recovered.

Apparent total clearance (CL) and volume of distribution (V_d) of ibuprofen was calculated from the following expressions:

$$\mathbf{CL = F.Dose/AUC} \qquad \text{(Eqn. 3.4)}$$

$$\mathbf{V_d = F.Dose/\lambda_z.AUC} \qquad \text{(Eqn. 3.5)}$$

where fraction of the dose reaching systemic circulation (F) can be assumed to be unity. This is a reasonable, as the bioavailability of (*R*)- and (*R,S*)-ibuprofen has been shown to be greater than 92% in comparative oral and intravenous (60-minute infusion) dosing studies (Hall *et al.*, 1993). As a consequence of chiral inversion, the dose of (*S*)-ibuprofen applicable to equations 3.4 and 3.5 must include the amount formed from metabolic chiral inversion of the *R*-enantiomer, this can be estimated using:

$$\mathbf{(\mathit{S})\text{-ibuprofen "dose"} = (1 + F_{inv}).200\ mg} \qquad \text{(Eqn. 3.6)}$$

Assuming all of a given metabolite formed is eliminated via renal excretion, the formation clearance (CL_f) of the major metabolites of ibuprofen, i.e. the stereoisomeric

forms of hydroxyibuprofen, carboxyibuprofen and ibuprofen glucuronide, can be estimated by:

$$\mathbf{CL_f = Ae_{(p)}/AUC_{parent}} \qquad \text{(Eqn. 3.7)}$$

where $Ae_{(p)}$ is the total urinary excretion (unconjugated plus conjugated) of the metabolite and AUC_{parent} is the AUC of the enantiomer from which the product was derived (Rudy *et al.*, 1995).

Free enantiomer concentrations were calculated by multiplying the respective mean unbound fraction by the total (bound and unbound) serum concentrations. The half-life ($t_{1/2,z\,u}$) and elimination rate constant ($\lambda_{z\,u}$) for each unbound concentration-time profile were determined by linear regression of the log-linear portion of the unbound enantiomer concentration versus time curves. The AUC for unbound drug (AUC_u) was calculated as:

$$\mathbf{AUC_u = unbound\ fraction \times AUC} \qquad \text{(Eqn. 3.8)}$$

Total body clearance (CL_u) and volume of distribution (V_{du}) based on unbound concentrations were calculated as before:

$$\mathbf{CL_u = F.Dose/AUC_u} \qquad \text{(Eqn. 3.9)}$$

$$\mathbf{V_{du} = CL_u/\lambda_{z\,u}} \qquad \text{(Eqn. 3.10)}$$

The unbound inversion clearance of (*R*)-ibuprofen ($CL^R_{inv,u}$) can be calculated from:

$$\mathbf{CL^R_{inv,u} = Finv.\ CL^R_u} \qquad \text{(Eqn.3.11)}$$

The unbound clearance of (*R*)-ibuprofen via non-inversion pathways ($CL^R_{other,\,u}$) can be established from:

$$\mathbf{CL^R_{other,u} = CL^R_u - CL^R_{inv,u}} \qquad \text{(Eqn.3.12)}$$

The mean pharmacokinetic parameters derived from the total and unbound serum data and urinary excretion data for the enantiomers of ibuprofen and hydroxyibuprofen and the stereoisomers of carboxyibuprofen were tested for stereoselective differences using the Student's t-test for paired samples in both age groups. Age-associated differences in the enantiospecific pharmacokinetic data and urinary data were assessed using the Student's t-test for independent samples.

3.3. Results

3.3.1 Serum kinetics

Enantioselective differences

The mean serum concentration-time profiles for the enantiomers of ibuprofen following the oral administration of 400 mg of the racemate to eight healthy young volunteers are shown in Figure 3.1, and the individual serum level data is tabulated in Appendix 2. The pharmacokinetic parameters derived from the serum concentrations are presented in Table 3.1. Following administration of 400 mg of the racemate, the serum levels of the two enantiomers of ibuprofen were indifferent during the initial four hour post-dose period, which is reflected in values for C_{max} and T_{max} displaying close correlation between the two enantiomers (Table 3.1). Subsequently, the serum concentrations of the *S*-enantiomer consistently exceeded those of its antipode such that the difference in enantiomeric concentrations reached statistical significance beyond 6 hours. This gradual enrichment of (*S*)-ibuprofen in serum during the elimination phase is also apparent from the mean half-live of the *S*-enantiomer being significantly greater than that of the *R*-antipode ($p < 0.05$; Table 3.1). These findings are consistent with previously reported data (Van Giessen and Kaiser, 1975; Lee *et al.*, 1985; Cox *et al.*, 1988; Avgerinos and Hutt, 1990; Tan, 1996; Suri *et al.*, 1997). The longer $t_{1/2,z}$ for the *S*-enantiomer was primarily due to the significantly larger volume of distribution of this enantiomer; with enantioselective total clearance, favouring the faster clearance of (*S*)-ibuprofen rather than its *R*-antipode, playing a less prominent role (Table 3.1). In addition, the AUC of the *S*-enantiomer was significantly greater than that of the *R*-enantiomer.

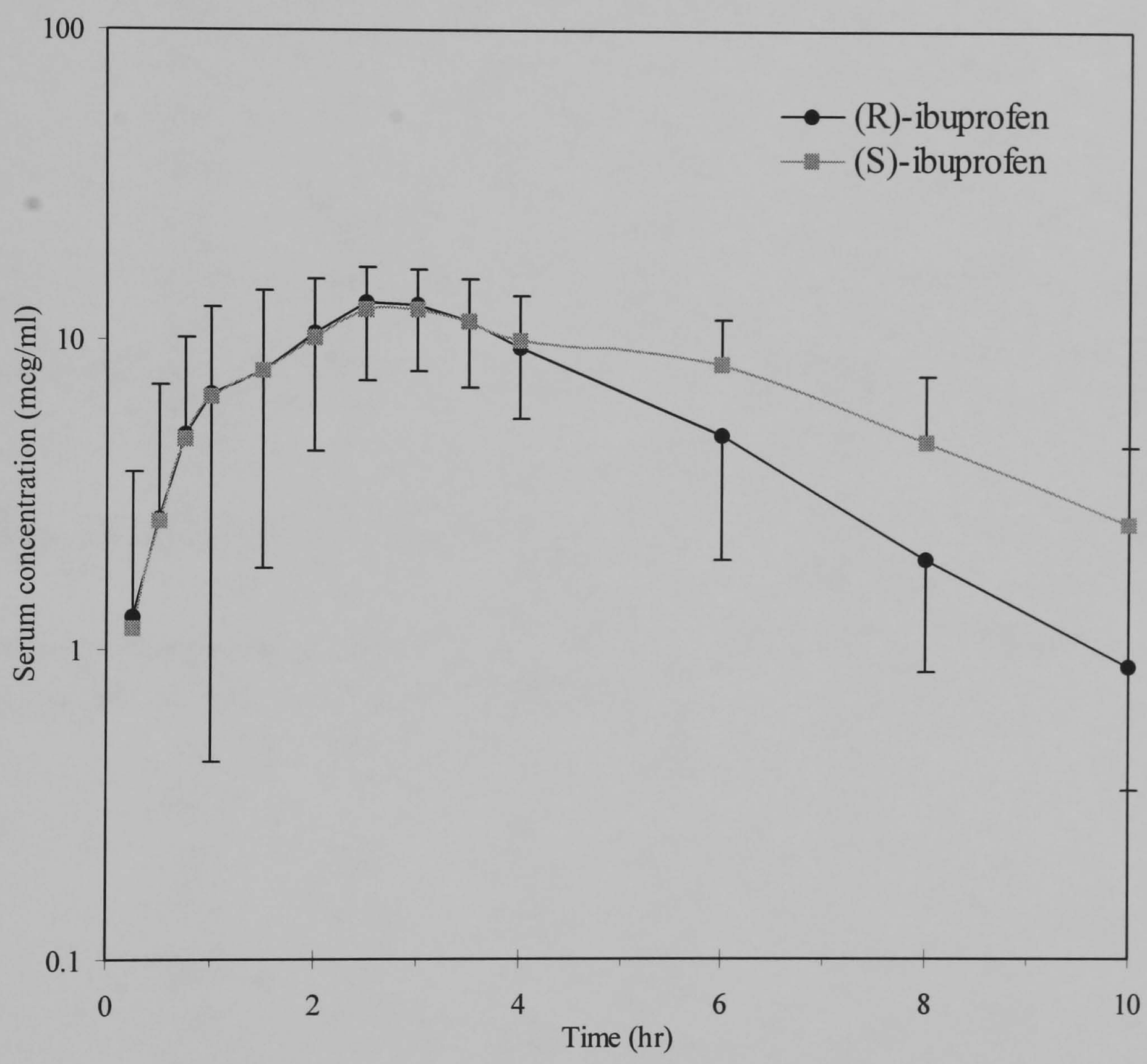


Figure 3.1: Mean serum concentration-time profiles for ibuprofen enantiomers following the oral administration of (*R,S*)-ibuprofen (400 mg) to eight healthy young volunteers (mean ± s.d.).

Table 3.1: Pharmacokinetic parameters of ibuprofen enantiomers following the oral administration of the racemic drug (400 mg) to eight healthy young volunteers.

				(*R*)-ibuprofen			
Subject Code	C_{max} (mcg/ml)	T_{max} (hr)	$t_{1/2,z}$ (hr)	AUC (mcg/ml hr)	CL (ml/min)	V_d (L)	F_{inv}
Iy1	16.1	1.0	1.6	67.3	49.5	7.0	0.62
Iy2	15.2	3.0	2.1	67.5	49.4	8.8	0.66
Iy3	14.9	1.5	1.4	55.3	60.3	7.0	0.74
Iy4	15.8	2.0	2.0	75.3	44.3	7.5	0.69
Iy5	23.0	2.5	1.4	80.6	41.4	4.9	0.66
Iy6	11.3	6.0	1.2	45.0	74.1	8.0	0.73
Iy7	11.6	2.5	1.5	42.9	77.7	9.8	0.68
Iy8	17.6	2.5	1.3	57.1	58.4	6.4	0.65
Mean	15.7	2.6	1.6	61.4	56.9	7.4	0.68
s.d.	3.7	1.5	0.3	13.6	13.4	1.5	0.04
C.V. %	23.4	57.4	20.8	22.2	23.5	20.1	6.0
				(*S*)-ibuprofen			
Iy1	16.8	1.0	1.8	71.6	75.4	11.8	
Iy2	16.1	3.5	2.1	77.2	71.7	12.9	
Iy3	16.2	2.0	2.6	98.5	58.9	13.4	
Iy4	14.9	2.0	2.5	84.3	66.8	14.2	
Iy5	16.8	2.5	2.4	76.9	72.0	14.9	
Iy6	16.1	6.0	3.6	78.2	73.7	22.9	
Iy7	13.7	3.5	2.3	75.5	74.2	14.4	
Iy8	14.1	3.0	2.2	65.9	83.5	16.0	
Mean	15.6	2.9	2.4	78.5	72.0	15.1	
s.d.	1.2	1.5	0.5	9.7	7.1	3.4	
C.V. %	7.7	51.0	21.8	12.3	9.8	22.7	
p (*R* vs. *S*) *	N.S.	N.S.	$p<0.05$	$p<0.05$	$p<0.05$	$p<0.001$	

** Comparison between the means for the enantiomers of ibuprofen were carried out using Student's t-test for paired samples (N.S. = $p>0.05$).*

The fraction unbound of the individual *R*- and *S*-enantiomers of ibuprofen for each volunteer was determined *ex vivo* following the replicate analysis (n =5) of a pooled serum sample which incorporated samples selected along the absorption phase, at C_{max} when ibuprofen and metabolite concentrations would be high and during the elimination phase (Table 3.2). Such an approach is reasonable since nonlinear protein binding for the enantiomers of ibuprofen is not expected to be issue at the relatively low dose and serum concentrations of this study (Evans *et al.*, 1989, 1990; Hall *et al.*, 1993).

Table 3.2: Percentage unbound of ibuprofen enantiomers following the oral administration of the racemic drug (400 mg) to eight healthy young volunteers (mean ± s.d.; n=5).

	% unbound	
Subject	***R*-isomer**	***S*-isomer**
Code	**mean ± s.d.**	**mean ± s.d.**
Iy1	0.25 ± 0.048	0.48 ± 0.041
Iy2	0.25 ± 0.042	0.43 ± 0.050
Iy3	0.19 ± 0.024	0.41 ± 0.024
Iy4	0.21 ± 0.031	0.48 ± 0.029
Iy5	0.21 ± 0.022	0.54 ± 0.045
Iy6	0.21 ± 0.040	0.47 ± 0.026
Iy7	0.24 ± 0.018	0.38 ± 0.037
Iy8	0.23 ± 0.009	0.45 ± 0.008
Mean ± s.d.	0.22 ± 0.022	0.46 ± 0.049

The percentage unbound of the *R*-enantiomer was significantly less than that of the *S*-enantiomer in all volunteers, with the mean values of 0.22 and 0.46 % for (*R*)- and (*S*)-ibuprofen respectively comparing favourably with values of 0.33 and 0.55 % obtained by Evans *et al.* (1990). The slight differences may be in part due to overestimation of the unbound fractions in the previous study as the values had not been corrected for volume shifts that occur during equilibrium dialysis (Evans *et al.*, 1990). Inter-subject variation in the enantiomeric binding ratio (percent unbound *S/R*) was large, varying approximately 60 % between 1.58 and 2.61, with a mean value of 2.09 which is within the range of values previously reported in *in vitro* studies (Evans *et al.*, 1990; Paliwal *et al.*, 1993; Smith *et al.*, 1994). The significance of the enantioselective protein binding in favour of the *R*-enantiomer is apparent from the serum unbound concentration-time curves, where the free serum levels of (*S*)-ibuprofen always

exceeded those of its antipode such that the difference reached statistical significance at all time points beyond 30 minutes post dosing (Figure 3.2).

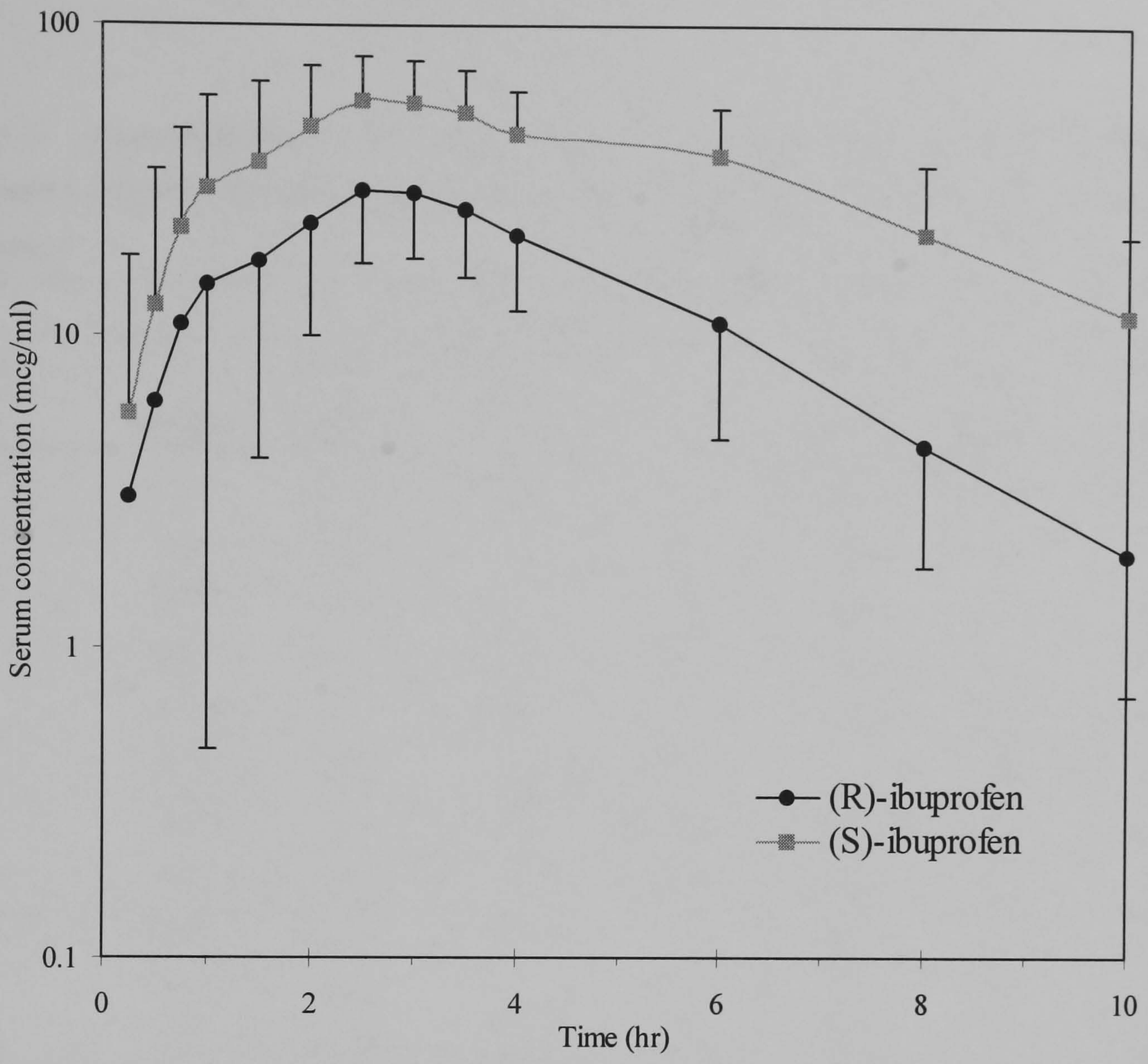


Figure 3.2: Mean serum unbound concentration-time profiles for ibuprofen enantiomers following the oral administration of (*R,S*)-ibuprofen (400 mg) to eight healthy young volunteers (mean ± s.d.).

The pharmacokinetic parameters obtained from the analysis of unbound serum concentrations for the young volunteers are presented in Table 3.3. When plasma protein binding is taken into account the significant enantioselectivity in volume of distribution disappears, but enantioselective differences in AUC_u and $t_{1/2,z}$, with the *S*-enantiomer displaying a higher AUC_u and longer $t_{1/2,z}$ are still observed. In contrast, unbound clearance exhibits enantioselectivity opposite to that of total clearance, with preference for the *R*-enantiomer rather than the *S*-enantiomer. Examination of the unbound clearance data for (*R*)-ibuprofen shows that this enantiomer is cleared predominately via inversion to its antipode, accounting for 69 % of the total unbound clearance. The unbound clearance of (*R*)-ibuprofen via other pathways was

approximately half the unbound clearance of (*S*)-ibuprofen, indicating that clearance through the oxidative and glucuronidation pathways was stereoselective for the *S*-enantiomer (Table 3.3).

Table 3.3: Pharmacokinetic parameters based on unbound concentrations of ibuprofen enantiomers following the oral administration of the racemic drug (400 mg) to eight healthy young volunteers.

				unbound (*R*)-ibuprofen			
Subject Code	**% unbound**	**AUCu (mg/ml hr)**	**$t_{1/2z,u}$ (hr)**	**V_{du} (L)**	**CL_u (L/min)**	**$CL_{inv,u}$ (L/min)**	**$CL_{other,u}$ (L/min)**
Iy1	0.25	0.17	1.6	2796	19.8	12.3	7.5
Iy2	0.25	0.17	2.1	3523	19.8	13.0	6.7
Iy3	0.19	0.11	1.4	3708	31.7	23.5	8.3
Iy4	0.21	0.16	2.0	3559	21.1	14.5	6.5
Iy5	0.21	0.17	1.4	2319	19.7	13.0	6.7
Iy6	0.21	0.09	1.2	3787	35.3	25.8	9.5
Iy7	0.24	0.10	1.5	4092	32.4	22.0	10.4
Iy8	0.23	0.13	1.3	2791	25.4	16.5	8.9
Mean	0.22	0.14	1.5	3322	25.6	17.6	8.1
s.d.	0.02	0.03	0.3	612	6.5	5.4	1.4
C.V. %	9.83	24.82	20.3	18	25.5	30.5	17.8
				unbound (*S*)-ibuprofen			
Iy1	0.48	0.34	1.8	2449	15.7		
Iy2	0.43	0.33	2.1	3002	16.7		
Iy3	0.41	0.40	2.6	3270	14.4		
Iy4	0.48	0.40	2.5	2965	13.9		
Iy5	0.54	0.42	2.4	2757	13.3		
Iy6	0.47	0.37	3.6	4863	15.7		
Iy7	0.38	0.29	2.3	3802	19.5		
Iy8	0.45	0.30	2.2	3581	18.6		
Mean	0.46	0.36	2.4	3336	16.0		
s.d.	0.05	0.05	0.5	755	2.2		
C.V. %	10.83	13.66	21.8	23	13.7		
p (*R* vs. *S*) *	p<0.001	p<0.001	p<0.05	N.S.	p<0.01		

** Comparison between the means for the enantiomers of ibuprofen were carried out using Student's t-test for paired samples (N.S. = p>0.05).*

The mean (*R*)- and (*S*)-ibuprofen serum concentration-time profiles following the administration of the racemic drug to eight healthy elderly volunteers are shown in Figure 3.3, and the individual serum level data is tabulated in Appendix 3. The pharmacokinetic parameters calculated from these data are presented in Table 3.4. In contrast to the young group, the serum levels of the *R*-enantiomer exceeded those of the S-antipode for the initial five hours following administration of the racemate to elderly volunteers; beyond this period the slower decline of (*S*)-ibuprofen, as also observed with the young, resulted in comparatively higher levels of the *S*-enantiomer in serum (Figure 3.3). The differences in the serum concentration of the two enantiomers were statistically significant at all sampling time after the initial 15 minutes post dose. The mean pharmacokinetic parameters determined for the elderly volunteers displayed similar enantioselective differences in CL, V_d and $t_{1/2,z}$ as those previously observed in the young, with the *S*-enantiomer having a greater CL and V_d and a longer $t_{1/2,z}$. A statistically significant difference in AUC was not observed with the elderly, although a trend towards a larger AUC for the *S*-enantiomer was apparent. Furthermore, the predominance of (*R*)-ibuprofen in serum during the absorption phase is reflected in the *R*-enantiomer displaying a significantly greater C_{max} than the *S*-antipode in this age group (Table 3.4).

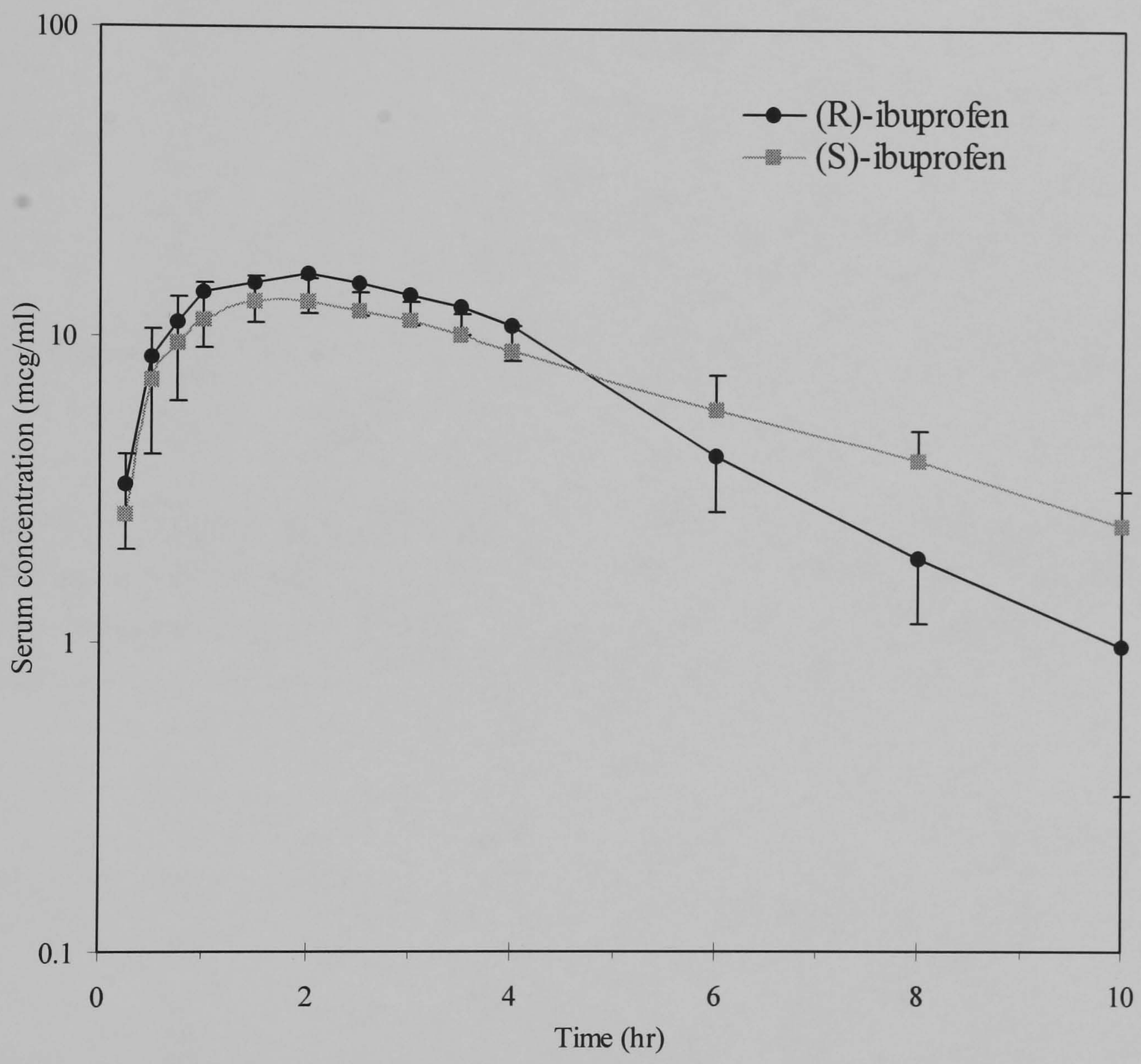


Figure 3.3: Mean serum concentration-time profiles for ibuprofen enantiomers following the oral administration of (*R,S*)-ibuprofen (400 mg) to eight healthy elderly volunteers (mean ± s.d.).

Table 3.4: Pharmacokinetic parameters of ibuprofen enantiomers following the oral administration of the racemic drug (400 mg) to eight healthy elderly volunteers.

				(*R*)-ibuprofen			
Subject Code	C_{max} (mcg/ml)	T_{max} (hr)	$t_{1/2,z}$ (hr)	AUC (mcg/ml hr)	CL (ml/min)	V_d (L)	F_{inv}
Ie1	19.3	2.0	2.8	95.9	34.8	8.3	0.65
Ie2	14.8	1.5	1.7	77.8	42.8	6.4	0.71
Ie3	14.4	1.0	1.8	75.0	44.4	7.0	0.67
Ie4	15.5	2.0	1.2	71.4	46.7	4.9	0.72
Ie5	11.5	1.5	1.8	54.9	60.7	9.4	0.66
Ie6	12.0	2.5	1.7	49.8	66.9	10.1	0.64
Ie7	20.4	2.0	1.1	78.1	42.7	3.9	0.62
Ie8	21.4	1.5	1.6	109.4	30.5	4.3	0.61
Mean	16.2	1.8	1.7	76.5	46.2	6.8	0.66
s.d.	3.8	0.5	0.5	19.5	12.2	2.3	0.04
C.V. %	23.4	26.5	30.0	25.5	26.4	34.5	6.0
p(Y vs. E) †	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
				(*S*)-ibuprofen			
Ie1	16.5	1.5	3.9	96.3	57.1	19.3	
Ie2	14.0	2.0	4.3	101.7	56.0	20.9	
Ie3	14.7	1.0	2.8	88.1	63.2	15.3	
Ie4	14.1	2.0	2.9	96.6	59.4	14.9	
Ie5	11.4	1.5	4.1	61.9	89.4	31.7	
Ie6	10.3	2.5	2.5	55.1	99.2	21.5	
Ie7	13.2	2.0	2.7	72.8	74.2	17.3	
Ie8	15.7	2.0	3.0	99.4	54.0	14.0	
Mean	13.7	1.8	3.3	84.0	69.1	19.4	
s.d.	2.1	0.5	0.7	18.2	17.0	5.7	
C.V. %	15.1	25.3	21.6	21.7	24.6	29.5	
p(R vs. S) *	p<0.05	N.S.	p<0.001	N.S.	p<0.0001	p<0.0001	
p(Y vs. E) †	p<0.05	N.S.	p<0.05	N.S.	N.S.	N.S.	

** Comparison between the means for the enantiomers of ibuprofen were carried out using Student's t-test for paired samples (N.S. = p>0.05).* † *Comparison between respective means for young and elderly volunteers were carried out using Student's t-test for independent samples (N.S. = p>0.05).*

The serum protein binding of ibuprofen in the elderly volunteers displayed the same enantioselectivity as that observed in the young group, with mean percentage unbound values for (*R*)- and (*S*)-ibuprofen of 0.22 and 0.53 % respectively (Table 3.5). High inter-subject variability in the enantiomeric binding ratio (unbound *S/R*) is also evident in this age group with values ranging from 1.69 to 3.23 around a mean value of 2.48. As observed with the young, the stereoselective protein binding is paralleled with serum concentrations of the free-form of (*S*)-ibuprofen being between 2 to 6 fold greater than those of the corresponding *R*-antipode concentrations (Figure 3.4).

Table 3.5: Percentage unbound of ibuprofen enantiomers following the oral administration of the racemic drug (400 mg) to eight healthy elderly volunteers (mean ± s.d.; n=5).

	% unbound	
Subject	***R*-isomer**	***S*-isomer**
Code	**mean ± s.d.**	**mean ± s.d.**
Ie1	0.21 ± 0.017	0.57 ± 0.042
Ie2	0.33 ± 0.061	0.56 ± 0.054
Ie3	0.24 ± 0.010	0.57 ± 0.015
Ie4	0.25 ± 0.044	0.53 ± 0.063
Ie5	0.19 ± 0.028	0.50 ± 0.036
Ie6	0.17 ± 0.007	0.46 ± 0.023
Ie7	0.25 ± 0.047	0.56 ± 0.048
Ie8	0.15 ± 0.019	0.49 ± 0.034
Mean ± s.d.	0.22 ± 0.057	0.53 ± 0.042

The pharmacokinetic parameters base on unbound serum concentrations displayed the same enantioselectivity between (*R*)- and (*S*)-ibuprofen as found with the young group; i.e. the *S*-enantiomer has a significantly longer $t_{1/2,z\ u}$, reduced CL_u and a larger AUC_u than the *R*-antipode (Table 3.6). The clearance pattern observed in the young group is also still evident in the elderly, with (*R*)-ibuprofen cleared primarily via chiral inversion and clearance through other general metabolic pathways exhibiting substrate stereoselectivity in favour of (*S*)-ibuprofen.

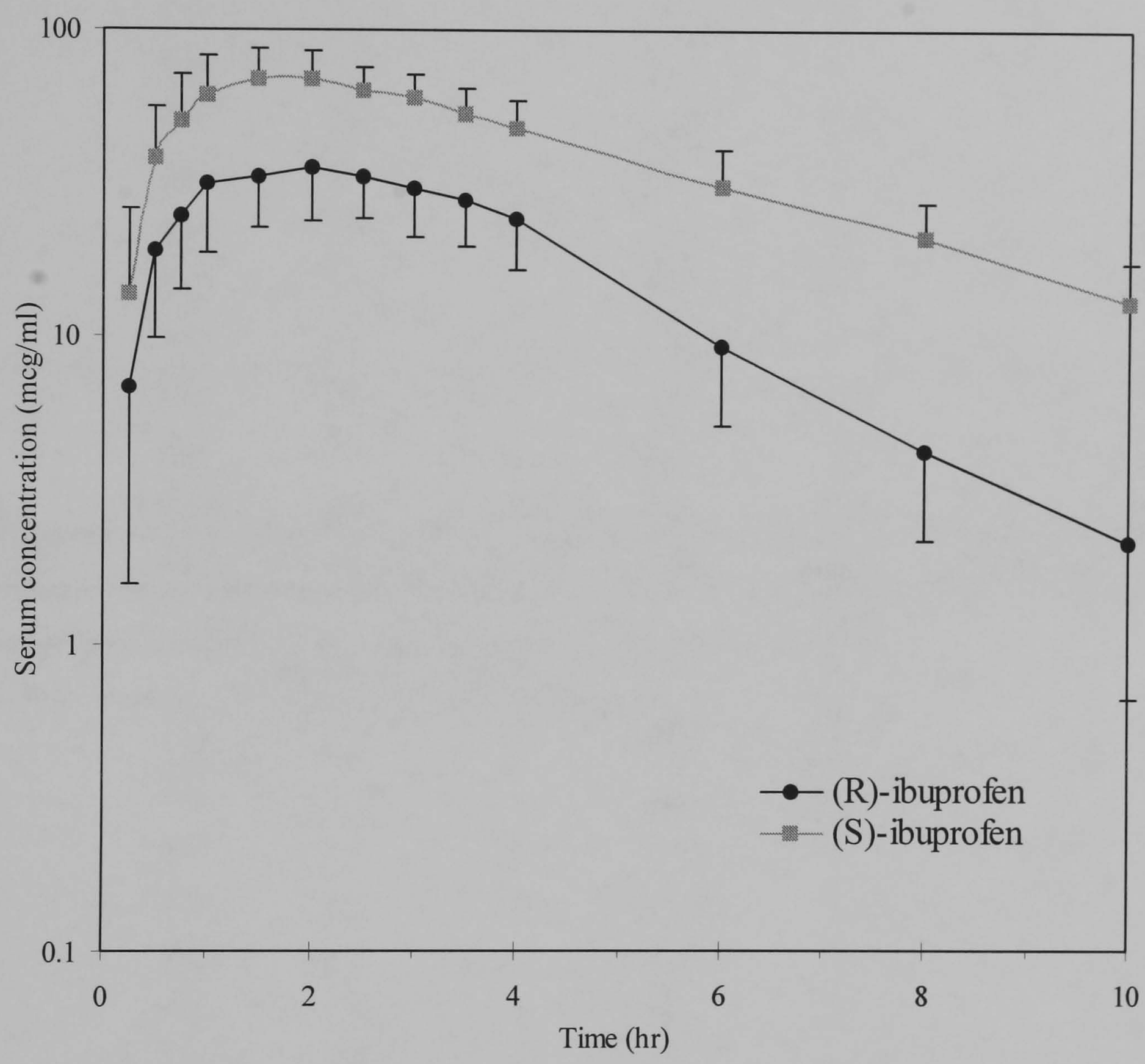


Figure 3.4: Mean serum unbound concentration-time profiles for ibuprofen enantiomers following the oral administration of (*R,S*)-ibuprofen (400 mg) to eight healthy elderly volunteers (mean ± s.d.).

Table 3.6: Pharmacokinetic parameters based on unbound concentrations of ibuprofen enantiomers following the oral administration of the racemic drug (400 mg) to eight healthy elderly volunteers.

				unbound (*R*)-ibuprofen			
Subject Code	% unbound	AUCu (mg/ml hr)	$t_{1/2z,u}$ (hr)	V_{du} (L)	CL_u (L/min)	$CL_{inv,u}$ (L/min)	$CL_{other,u}$ (L/min)
Ie1	0.21	0.20	2.8	3941	16.6	10.8	5.8
Ie2	0.33	0.26	1.7	1945	13.0	9.2	3.8
Ie3	0.24	0.18	1.8	2918	18.5	12.4	6.1
Ie4	0.25	0.18	1.2	1956	18.7	13.5	5.2
Ie5	0.19	0.10	1.9	5257	32.0	21.1	10.9
Ie6	0.17	0.08	1.8	5966	39.4	25.2	14.2
Ie7	0.25	0.20	1.1	1567	17.1	10.6	6.5
Ie8	0.15	0.16	1.6	2884	20.3	12.4	7.9
Mean	0.22	0.17	1.7	3304	21.9	14.4	7.5
s.d.	0.06	0.06	0.5	1617	9.0	5.7	3.4
C.V. %	25.39	33.86	29.3	49	40.9	39.4	45.1
p(Y vs. E) †	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
				unbound (*S*)-ibuprofen			
Ie1	0.57	0.55	4.0	3435	10.0		
Ie2	0.56	0.57	4.3	3726	10.0		
Ie3	0.57	0.50	2.8	2678	11.1		
Ie4	0.53	0.51	2.9	2792	11.2		
Ie5	0.50	0.31	4.1	6408	17.9		
Ie6	0.46	0.25	2.6	4874	21.6		
Ie7	0.56	0.41	2.8	3246	13.3		
Ie8	0.49	0.49	3.0	2862	11.0		
Mean	0.53	0.45	3.3	3753	13.3		
s.d.	0.04	0.12	0.7	1284	4.2		
C.V. %	7.94	25.73	20.9	34	31.9		
p(R vs. S) *	p<0.001	p<0.001	p<0.001	N.S.	p<0.0001		
p(Y vs. E) †	p<0.05	N.S.	p<0.05	N.S.	N.S.		

** Comparison between the means for the enantiomers of ibuprofen were carried out using Student's t-test for paired samples (N.S. = p>0.05).* † *Comparison between respective means for young and elderly volunteers were carried out using Student's t-test for independent samples (N.S. = p>0.05).*

Age-associated differences

Comparison between the young and elderly groups indicated age-related alterations in the stereoselectivity of ibuprofen disposition (Table 3.4). There were no differences in the pharmacokinetic parameters based on total serum concentrations for (*R*)-ibuprofen between the two age groups. But significant differences were observed in the case of the *S*-enantiomer indicating that the elderly have a lower C_{max} and a longer elimination half life for the active (*S*)-ibuprofen. There was also trends towards a larger V_d for the *S*-enantiomer in the elderly, but the difference between the two age groups did not achieve statistical significant ($p = 0.09$; Table 3.4).

Examination of the unbound fractions for the individual enantiomers of ibuprofen in the two age groups reveals that ageing is associated with significant changes in the extent of serum protein binding of (*S*)- but not (*R*)-ibuprofen; such that the mean enantiomeric ratio (percent unbound *S/R*) increased from 2.07 ± 0.33 in the young to 2.48 ± 0.47 in the elderly subjects (Tables 3.2 and 3.5). Pharmacokinetic parameters based on unbound serum concentrations displayed, as would be expected, no age-related differences for (*R*)-ibuprofen (Table 3.6). In the case of unbound (*S*)-ibuprofen a significant difference was only evident for $t_{1/2,z\ u}$ between the two age groups which mirrored the age-related difference observed in the half-life of (*S*)-ibuprofen based on total serum concentrations, this is not surprising since in the presence of a constant fraction unbound values of half-life are not influenced by the use of unbound drug concentrations. However, it is also noteworthy that there is a trend towards a larger AUC_u for (*S*)-ibuprofen in the elderly, but the difference between the two age groups did not achieve statistical significant ($p = 0.06$; Table 3.6).

3.3.2 Urinary excretion

Enantioselective differences

The urinary excretion data for ibuprofen and its metabolites following the oral administration of the drug to young volunteers is summarised in Table 3.7 and the individual data tabulated in Appendices 4 to 6. The calculated inversion fractions and formation clearances are tabulated in Tables 3.1 and 3.8. The total amount of ibuprofen and metabolites recovered in the 0-24 hour urine collection period was 72.8 ± 11.8 % which compares favourably with previously published values of 70 to 79 % (Evans *et al.*, 1990; Smith *et al.*, 1994; Rudy *et al.*, 1995; Scheuerer *et al.*, 1998). The principle pathway of ibuprofen elimination is via oxidative metabolism to form hydroxy- and carboxyibuprofen with recovery values of 21.2 and 39.4 % respectively. Elimination as ibuprofen conjugates is a relatively minor pathway, accounting for only 12.2 % of the administered dose excreted in urine (Evans *et al.*, 1990; Smith *et al.*, 1994; Scheuerer *et al.*, 1998).

The fraction of the administered dose of (*R*)-ibuprofen inverted to (*S*)-ibuprofen (F_{inv}), determined using urinary data, had a mean value of 0.68 which shows good agreement with previously reported values calculated by either separate administration of the *R*-enantiomer, by the urinary metabolite method, or by administration of a pseudoracemate (Lee *et al.*, 1985; Rudy *et al.*, 1991; Smith *et al.*, 1994). The range of F_{inv} values was from 0.62 to 0.74 indicating limited inter-subject variability in the extent of chiral inversion, which is consistent with the observations made previously based on indirect evidence (Avgerinos and Hutt, 1990).

Ibuprofen was present in urine almost exclusively as the acyl glucuronide of the *S*-enantiomer (recovery ratio *S/R* = 11.5; Table 3.7). This appears to be as a consequence not only of the metabolic chiral inversion of (*R*)- to (*S*)-ibuprofen but also the stereoselective clearance of (*S*)-ibuprofen as the glucuronide metabolite, which is indicated by the enantioselectivity observed in the formation clearances of ibuprofen glucuronides (Table 3.8). The presence of small amounts of free (*R*)- and (*S*)-ibuprofen in the urine, accounting for about 1 % of the dose, is probably as a result of spontaneous hydrolysis of the glucuronide during sample manipulation rather than excretion of the unchanged drug (Lockwood *et al.*, 1983; Lee *et al.*, 1985; Evans, 1992).

Table 3.7: Urinary excretion (0-24 hr) of ibuprofen and its metabolites following the oral administration of the racemic drug (400 mg) to eight healthy young volunteers (data expressed as a mean percentage of the administered dose).

	***R*-isomer**	***S*-isomer**
Analyte	**mean ± s.d.**	**mean ± s.d.**
Ibuprofen		
free:	0.06 ± 0.03	0.96 ± 0.46 *
conjugate:	0.89 ± 0.16	10.24 ± 2.08 *
total	0.95 ± 0.16	11.20 ± 2.15 *
Hydroxyibuprofen		
free:	2.69 ± 0.80	7.82 ± 2.13 *
conjugate:	0.62 ± 0.28	10.11 ± 1.48 *
total	3.31 ± 0.82	17.92 ± 1.89 *
Carboxyibuprofen, *2'R-*		
free:	3.40 ± 0.40	10.76 ± 2.40 *
conjugate:	1.20 ± 0.62	3.51 ± 1.93 *
total	4.60 ± 0.70	14.27 ± 2.60 *
Carboxyibuprofen, *2'S-*		
free:	2.34 ± 0.38	12.86 ± 2.73 *
conjugate:	0.51 ± 0.30	4.79 ± 2.09 *
total	2.84 ± 0.55	17.65 ± 2.91 *
Total Recovery	11.71 ± 1.75	61.04 ± 7.04 *

** $p < 0.05$ for comparison between the means of enantiomers using Student's t-test for paired samples.*

Table 3.8: Formation clearances (ml/min) for (a) ibuprofen glucuronides, hydroxyibuprofen and carboxyibuprofen and (b) individual carboxyibuprofen stereoisomers following the oral administration of the racemic drug (400 mg) to eight healthy young volunteers.

a) Subject	conjugate		hydroxyibuprofen		carboxyibuprofen	
Code	***R***	***S***	***R***	***S***	***2'S,2R + 2'R,2R***	***2'R,2S + 2'S,2S***
Iy1	1.10	9.99	4.32	15.64	7.70	26.15
Iy2	0.88	7.56	2.37	13.62	7.82	25.86
Iy3	1.22	9.50	2.89	12.38	7.23	19.94
Iy4	0.97	9.24	2.41	13.76	5.91	21.79
Iy5	0.68	10.91	3.70	17.20	8.13	34.90
Iy6	1.29	9.55	4.44	17.89	10.77	34.04
Iy7	1.04	6.74	5.31	16.46	11.31	29.25
Iy8	1.32	13.13	4.37	15.83	7.96	27.29
Mean	1.06	9.58	3.73	15.35	8.35	27.40
s.d.	0.22	1.96	1.07	1.92	1.80	5.28
C.V. %	20.53	20.43	28.79	12.50	21.57	19.25
p (*R* vs. *S*) *	p<0.0005		p<0.0005		p<0.0005	

b) Subject	carboxyibuprofen stereoisomers			
Code	***2'S,2R***	***2'R,2R***	***2'R,2S***	***2'S,2S***
Iy1	2.69	5.00	11.94	14.21
Iy2	3.22	4.60	11.00	14.86
Iy3	2.57	4.67	9.12	10.82
Iy4	2.09	3.82	9.78	12.00
Iy5	3.19	4.94	16.17	18.73
Iy6	4.27	6.50	14.72	19.32
Iy7	3.90	7.41	13.71	15.53
Iy8	3.55	4.41	11.44	15.85
Mean	3.19	5.17	12.24	15.17
s.d.	0.72	1.19	2.44	2.94
C.V. %	22.66	22.94	19.97	19.38
p(2'R vs.2' S) *	p<0.0005		p<0.0005	

** Comparison between the means of the enantiomers of ibuprofen and hydroxyibuprofen and that of the individual stereoisomers of carboxyibuprofen and the stereoisomers of carboxyibuprofen derived from (R)- and (S)-ibuprofen were performed using a Student's t-test for paired samples (N.S. = $p > 0.05$).*

NOTE: data for carboxyibuprofen are presented for both the individual stereoisomers and also for the sum of the stereoisomers produced from an individual isomer of ibuprofen, i.e. the two diastereoisomers 2'S,2R and 2'R,2R arise via oxidation of (R)-ibuprofen whereas 2'R,2S and 2'S,2S arise from oxidation of the S-enantiomer.

Clearance of ibuprofen via oxidative metabolic pathways, i.e. via the formation of hydroxyibuprofen and carboxyibuprofen, also display substrate stereoselectivity towards (*S*)-ibuprofen as reflected in the formation clearances, which were significantly greater for the metabolites derived from the *S*-enantiomer compared to the *R*-antipode (*S/R*: hydroxyibuprofen = 4.1 and carboxyibuprofen = 3.3; Table 3.8). Furthermore, for both (*R*)- and (*S*)-ibuprofen the formation clearances were greater for carboxyibuprofen rather than hydroxyibuprofen indicating that oxidation has preference for the 3'-position rather than 2'-position of the isobutyl group. Thus, clearance via oxidative metabolism exhibits stereoselectivity in favour of the *S*-enantiomer and regioselectivity in favour of carboxyibuprofen formation, this pattern is reflected in the metabolite excretion data (Table 3.7). It is also apparent that formation of the carboxyibuprofen stereoisomers displays product, as well as substrate, stereoselectivity with the predominantly formed diastereoisomer having the same configuration at the metabolically introduced chiral centre as that in the substrate propionic acid moiety i.e. oxidation of (*R*)-ibuprofen favours (2'*R*,2*R*)-carboxyibuprofen formation (2'*R*,2*R*/2'*S*,2*R* = 1.6) and (*S*)-ibuprofen favours the formation of (2'*S*,2*S*)-carboxyibuprofen (2'*S*,2*S*/2'*R*,2*S* = 1.2; Table 3.8).

In contrast to the situation with the parent drug, substantial amounts of the hydroxy and carboxy metabolites were excreted in urine in the non-conjugated, as well as conjugated, form (Table 3.7). Interestingly, (*R*)-hydroxyibuprofen is predominantly recovered as the free metabolite (conjugate/free = 0.23) whereas (*S*)-hydroxyibuprofen was excreted mainly as the conjugate (conjugate/free = 1.3), indicating that conjugation of hydroxyibuprofen displays enantioselectivity in favour of the *S*-enantiomer. In the case of carboxyibuprofen, all four stereoisomers were present in urine predominantly as the free metabolite, which might be as predicted on the basis of its high polarity. The individual conjugate/free ratios of 0.35, 0.33, 0.22 and 0.37 for the 2'*R*,2*R*, 2'*R*,2*S*, 2'*S*,2*R* and 2'*S*,2*S* stereoisomers respectively, suggests that (2'*S*,2*R*)-carboxyibuprofen is a poorer substrate for conjugation than the other three stereoisomers (Table 3.7). This is most probably as a consequence of stereoselective conjugation since recently it has been reported that carboxyibuprofen, like ibuprofen and hydroxyibuprofen, undergoes conjugation with glucuronic acid to form β-1-*O*-acyl glucuronides at the carboxylic acid group of the propionic acid side chain and thus regioselectivity in conjugation is not an issue (Kepp *et al.*, 1997).

Metabolite formation clearances based on unbound rather than total serum concentrations of ibuprofen revealed more modest, but still significant, differences

between the enantiomers for all metabolic pathways (Table 3.9). Thus, the enantiomeric ratio (*S/R*) of the formation clearances for ibuprofen glucuronides decreased from 9.0 to 4.4 when calculated from total and unbound enantiomer data respectively. Similarly, the corresponding values for the hydroxy metabolite decreased from 4.1 to 2.0 and those for the sum of the carboxy diastereoisomers derived from a particular ibuprofen enantiomer decreased from 3.3 to 1.6. However, the product selectivity ratios, i.e. the ratio for the carboxyibuprofen stereoisomers derived from a single enantiomer of ibuprofen, remained constant at 1.62 and 1.24 for 2'*R*,2*R*/2'*S*,2*R* and 2'*S*,2*S*/2'*R*,2*S* respectively; which is not surprising since the influence of unbound fraction is negated in these ratios. Therefore, even though the relative stereoselectivity of the individual pathways is reduced when based on unbound formation clearances, the data indicates inherent stereoselectivity of the associated metabolic pathways. So, the overall stereochemical composition of the metabolites in urine is reflective of stereoselectivity in both protein binding and metabolism, and possibly also of renal excretion.

Table 3.9: Unbound formation clearances (L/min) for (a) ibuprofen glucuronides, hydroxyibuprofen and carboxyibuprofen and (b) individual carboxyibuprofen stereoisomers following the oral administration of the racemic drug (400 mg) to eight healthy young volunteers.

a)

Subject	conjugate		hydroxyibuprofen		carboxyibuprofen	
Code	*R*	*S*	*R*	*S*	*2'S,2R + 2'R,2R*	*2'R,2S + 2'S,2S*
Iy1	0.44	2.10	1.71	3.29	3.05	5.51
Iy2	0.35	1.77	0.94	3.19	3.11	6.05
Iy3	0.61	2.34	1.45	3.05	3.64	4.91
Iy4	0.45	1.95	1.13	2.90	2.78	4.59
Iy5	0.32	2.00	1.75	3.15	3.85	6.39
Iy6	0.64	2.02	2.22	3.78	5.39	7.19
Iy7	0.45	1.75	2.28	4.29	4.85	7.61
Iy8	0.58	1.88	1.92	3.48	3.50	6.00
Mean	0.48	1.98	1.68	3.39	3.77	6.03
s.d.	0.12	0.19	0.48	0.45	0.91	1.04
C.V. %	24.70	9.63	28.70	13.34	24.18	17.24
p (*R* vs. *S*) *	p<0.0005		p<0.0005		p<0.0005	

b)

Subject	carboxyibuprofen stereoisomers			
Code	*2'S,2R*	*2'R,2R*	*2'R,2S*	*2'S,2S*
Iy1	1.07	1.98	2.51	2.99
Iy2	1.28	1.83	2.57	3.48
Iy3	1.29	2.35	2.25	2.67
Iy4	0.98	1.80	2.06	2.53
Iy5	1.51	2.34	2.96	3.43
Iy6	2.13	3.25	3.11	4.08
Iy7	1.67	3.18	3.57	4.04
Iy8	1.56	1.94	2.51	3.48
Mean	1.44	2.33	2.69	3.34
s.d.	0.37	0.58	0.49	0.57
C.V. %	25.58	24.96	18.27	17.22
p(2'R vs.2' S) *	p<0.0005		p<0.0005	

** Comparison between the means of the enantiomers of ibuprofen and hydroxyibuprofen and that of the individual stereoisomers of carboxyibuprofen and the stereoisomers of carboxyibuprofen derived from (R)- and (S)-ibuprofen were performed using a Student's t-test for paired samples (N.S. = $p > 0.05$).*

NOTE: data for carboxyibuprofen are presented for both the individual stereoisomers and also for the sum of the stereoisomers produced from an individual isomer of ibuprofen, i.e. the two diastereoisomers 2'S,2R and 2'R,2R arise via oxidation of (R)-ibuprofen whereas 2'R,2S and 2'S,2S arise from oxidation of the S-enantiomer.

The urinary excretion data for ibuprofen and its metabolites for the elderly group is presented in Table 3.10 and the individual data tabulated in Appendices 7 to 9. The calculated inversion fraction and formation clearances are tabulated in Tables 3.4 and 3.11. Approximately 66.8 ± 15.6 % of the ibuprofen dose was recovered in urine as ibuprofen or its metabolites over the 0-24 hour collection period. The hydroxy and carboxy metabolites formed 19.1 and 35.2 % of the administered dose recovered in urine, with the parent drug accounting for only 12.4 % (Table 3.10). The mean value for the F_{inv} was 0.66 which is in close agreement with the value obtained in the young and in a similar manner, there was little variability in the extent of inversion between the elderly volunteers.

As with the young group, ibuprofen was excreted mainly as the acyl glucuronide of the *S*-enantiomer with an *S/R* recovery ratio of 11.0; which is paralleled with (*S*)-ibuprofen glucuronide having a significantly greater formation clearance than the *R*-antipode. Stereoselective clearance was also observed with the hydroxyibuprofen and carboxyibuprofen metabolites as reflected by the respective formation clearances, which were significantly greater for the metabolites derived from (*S*)-ibuprofen compared to those of the (*R*)-enantiomer (*S/R*: hydroxyibuprofen = 4.9, carboxyibuprofen = 3.5; Table 3.11). The formation of the carboxyibuprofen diastereoisomers displayed the same substrate related product stereoselectivity as that observed with the young volunteers, i.e. oxidation of (*R*)-ibuprofen preferentially yields (2'*R*,2*R*)-carboxy-ibuprofen (2'*R*,2*R*/2'*S*,2*R* = 1.5) and (*S*)-ibuprofen preferentially forms (2'*S*,2*S*)-carboxy-ibuprofen (2'*S*,2*S*/2'*R*,2*S* = 1.3; Table 3.11).

The urinary excretion of the oxidative products, as free metabolites or as conjugates, showed a similar stereochemical pattern to that observed in the young (Table 3.10). Stereoselective conjugation of hydroxyibuprofen is indicated by the recovery of the *S*-enantiomer primarily as the conjugate (conjugate/free = 1.36) and the *R*-antipode mainly in the unconjugated form (conjugate/free = 0.17). In the case of the carboxy metabolite, the individual conjugate/free ratios of 0.35, 0.33,0.23 and 0.37 for the 2'*R*,2*R*, 2'*R*,2*S*, 2'*S*,2*R* and 2'*S*,2*S* stereoisomers respectively, also suggests that (2'*S*,2*R*)-carboxyibuprofen is a poorer substrate for conjugation than the other three stereoisomers in this age group (Table 3.11).

In a similar manner to the young group, unbound formation clearances displayed more modest, but still significant, differences between the enantiomers (Table 3.12).

Table 3.10: Urinary excretion (0-24 hr) of ibuprofen and its metabolites following the oral administration of the racemic drug (400 mg) to eight healthy elderly volunteers (data expressed as a mean percentage of the administered dose).

	***R*-isomer**	***S*-isomer**
Analyte	**mean ± s.d.**	**mean ± s.d.**
Ibuprofen		
free:	0.09 ± 0.04	1.02 ± 0.70 *
conjugate:	0.94 ± 0.28	10.32 ± 3.42 *
total	1.04 ± 0.31	11.34 ± 3.86 *
Hydroxyibuprofen		
free:	2.57 ± 0.87	6.83 ± 2.17 *
conjugate:	0.43 ± 0.20	9.31 ± 2.27 *
total	3.00 ± 0.94	16.14 ± 3.86 *
Carboxyibuprofen, *2'R*-		
free:	3.24 ± 0.99	9.30 ± 2.35 *
conjugate:	1.13 ± 0.92	3.03 ± 3.16 *
total	4.37 ± 1.17	12.33 ± 2.98 *
Carboxyibuprofen, *2'S*-		
free:	2.46 ± 0.71	11.29 ± 3.07 *
conjugate:	0.57 ± 0.82	4.23 ± 3.60 *
total	3.02 ± 0.80	15.52 ± 3.42 *
Total Recovery	11.43 ± 2.69	55.33 ± 10.25 *

** $p < 0.05$ for comparison between the means of enantiomers using Student's t-test for paired samples. † p-value <0.05 for comparison between respective means for young and elderly volunteers using Student's t-test for independent samples*

Table 3.11: Formation clearances (ml/min) for (a) ibuprofen glucuronides, hydroxyibuprofen and carboxyibuprofen and (b) individual carboxyibuprofen stereoisomers following the oral administration of the racemic drug (400 mg) to eight healthy elderly volunteers.

a)

Subject	conjugate		hydroxyibuprofen		carboxyibuprofen	
Code	*R*	*S*	*R*	*S*	*2'S,2R + 2'R,2R*	*2'R,2S + 2'S,2S*
Ie1	0.52	7.66	2.02	10.55	6.44	23.97
Ie2	0.97	8.53	1.49	8.26	4.07	12.07
Ie3	0.70	6.33	1.49	7.05	4.67	15.60
Ie4	1.29	12.93	2.84	12.77	5.76	18.88
Ie5	1.82	15.29	4.09	19.07	9.39	32.89
Ie6	0.84	8.19	4.79	23.97	12.32	42.38
Ie7	0.82	8.18	2.72	13.86	6.19	21.88
Ie8	0.71	6.43	2.75	13.96	5.80	21.72
Mean	0.96	9.19	2.77	13.69	6.83	23.67
s.d.	0.42	3.20	1.18	5.59	2.72	9.75
C.V. %	43.30	34.84	42.37	40.87	39.81	41.17
p (*R* vs. *S*) *	p<0.0005		p<0.0005		p<0.0005	
p(Y vs. E) †	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

b)

Subject	carboxyibuprofen stereoisomers			
Code	*2'S,2R*	*2'R,2R*	*2'R,2S*	*2'S,2S*
Ie1	2.75	3.69	10.97	13.00
Ie2	1.81	2.26	5.19	6.88
Ie3	2.04	2.60	6.82	8.78
Ie4	1.97	3.79	8.57	10.30
Ie5	4.23	5.16	13.94	18.96
Ie6	4.44	7.87	19.37	23.01
Ie7	2.42	3.77	9.72	12.16
Ie8	2.48	3.32	9.33	12.39
Mean	2.77	4.06	10.49	13.19
s.d.	1.02	1.77	4.44	5.33
C.V. %	36.70	43.63	42.36	40.45
p(2'R vs.2' S) *	p<0.0005		p<0.0005	
p(Y vs. E) †	N.S.	N.S.	N.S.	N.S.

** Comparison between the means of the enantiomers of ibuprofen and hydroxyibuprofen and that of the individual stereoisomers of carboxyibuprofen and the stereoisomers of carboxyibuprofen derived from (R)- and (S)-ibuprofen were performed using a Student's t-test for paired samples (N.S. = p > 0.05).* † *Comparison between respective means for young and elderly volunteers were carried out using Student's t-test for independent samples (N.S. = p > 0.05).*

NOTE: data for carboxyibuprofen are presented for both the individual stereoisomers and also for the sum of the stereoisomers produced from an individual isomer of ibuprofen, i.e. the two diastereoisomers 2'S,2R and 2'R,2R arise via oxidation of (R)-ibuprofen whereas 2'R,2S and 2'S,2S arise from oxidation of the S-enantiomer.

Table 3.12: Unbound formation clearances (L/min) for (a) ibuprofen glucuronides, hydroxyibuprofen and carboxyibuprofen and (b) individual carboxyibuprofen stereoisomers following the oral administration of the racemic drug (400 mg) to eight healthy elderly volunteers.

a)

Subject	conjugate		hydroxyibuprofen		carboxyibuprofen	
Code	*R*	*S*	*R*	*S*	*2'S,2R + 2'R,2R*	*2'R,2S + 2'S,2S*
Ie1	0.25	1.34	0.97	1.85	3.09	4.20
Ie2	0.29	1.52	0.45	1.47	1.22	2.15
Ie3	0.29	1.12	0.62	1.24	1.93	2.75
Ie4	0.51	2.45	1.13	2.42	2.29	3.58
Ie5	1.00	3.05	2.25	3.81	5.15	6.57
Ie6	0.53	1.81	2.98	5.28	7.67	9.34
Ie7	0.32	1.45	1.06	2.46	2.42	3.88
Ie8	0.48	1.30	1.88	2.83	3.97	4.41
Mean	0.46	1.76	1.42	2.67	3.47	4.61
s.d.	0.25	0.66	0.87	1.33	2.10	2.32
C.V. %	53.45	37.86	61.57	49.85	60.48	50.23
p (R vs. *S)* *	p<0.0005		p<0.0005		p<0.0005	
p(Y vs. E) †	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

b)

Subject	carboxyibuprofen stereoisomers			
Code	*2'S,2R*	*2'R,2R*	*2'R,2S*	*2'S,2S*
Ie1	1.32	1.77	1.92	2.28
Ie2	0.54	0.68	0.93	1.23
Ie3	0.85	1.08	1.20	1.55
Ie4	0.78	1.50	1.62	1.95
Ie5	2.32	2.83	2.78	3.78
Ie6	2.77	4.90	4.27	5.07
Ie7	0.94	1.47	1.73	2.16
Ie8	1.70	2.27	1.89	2.51
Mean	1.40	2.06	2.04	2.57
s.d.	0.80	1.33	1.05	1.26
C.V. %	56.90	64.30	51.57	49.27
p(2'R vs.2' S) *	p<0.0005		p<0.0005	
p(Y vs. E) †	N.S.	N.S.	N.S.	N.S.

** Comparison between the means of the enantiomers of ibuprofen and hydroxyibuprofen and that of the individual stereoisomers of carboxyibuprofen and the stereoisomers of carboxyibuprofen derived from (R)- and (S)-ibuprofen were performed using a Student's t-test for paired samples (N.S. = p > 0.05).* † *Comparison between respective means for young and elderly volunteers were carried out using Student's t-test for independent samples (N.S. = p > 0.05).*

NOTE: data for carboxyibuprofen are presented for both the individual stereoisomers and also for the sum of the stereoisomers produced from an individual isomer of ibuprofen, i.e. the two diastereoisomers 2'S,2R and 2'R,2R arise via oxidation of (R)-ibuprofen whereas 2'R,2S and 2'S,2S arise from oxidation of the S-enantiomer.

Thus, the enantiomeric ratio (*S/R*) of the formation clearances for ibuprofen glucuronides decreased from 9.6 to 3.8 when calculated from total and unbound enantiomer data respectively. Likewise, the corresponding values for the hydroxy metabolite decreased from 4.9 to 1.9 and those for the sum of the carboxy diastereoisomers derived from a particular ibuprofen enantiomer decreased from 3.5 to 1.3.

Age-associated differences

A lower percentage of the dose, although not significant, was recovered over the 24 hour urine collection period in the elderly (66.8% vs. 72.7%). This appears to be primarily due to a modest decrease in the recovery of the oxidative products of (*S*)-ibuprofen, with the amounts of material excreted as the parent drug, both free and conjugated, and as *R*-configuration oxidative metabolites essentially invariant between the two age groups (Tables 3.7 and 3.10).

There was a trend for metabolite formation clearances based on total serum concentrations to be lower in the elderly volunteers (Table 3.8 and 3.11). But the differences did not achieve statistical significance, this could possibly be due to the large inter-subject variability in the values, especially within the elderly volunteer group. It is noteworthy, that for a given metabolic pathway the proportionate decrease in clearance with age was always to a lesser extent for the *S*-enantiomer. This pattern is probably reflective of the higher unbound fraction for (*S*)-ibuprofen in the elderly partially counteracting any age related decreases in the individual unbound formation clearances leading to less substantial changes in the associated formation clearances. Comparison of unbound formation clearances did suggest that the elderly volunteers were generally less able to metabolise (*R*)- and (*S*)-ibuprofen; however, the differences were largely trends with none reaching statistical significance. In contrast, to the observation with total formation clearances, decreases in unbound formation clearances were more apparent for metabolites derived from (*S*)-ibuprofen. For instance, the unbound formation clearances for (*R*)- and (*S*)-ibuprofen glucuronide were lower by 4.1 % and 11.0 % respectively in the elderly. In a similar manner, the corresponding values for (*R*)- and (*S*)-hydroxyibuprofen decreased by 15.5 % and 21.2 %, and those for the sum of the carboxy diastereoisomers derived from (*R*)- and (*S*)-ibuprofen decreased by 8.0 % and 23.6 %.respectively. Thus, it would appear that elimination of (*S*)-ibuprofen is more sensitive to decreased metabolic activity in the elderly and it is also interesting

to note that clearance via the glucuronidation pathway is least influenced by age. This pattern of age-related alteration in the metabolic activity is reflected in the elderly having a reduced unbound clearance for (*S*)-ibuprofen (Y: 16.0 vs. E:13.3 L/min), although the difference between the two age groups did not statistical significance.

3.3.3 Pharmacodynamic studies

The mean serum thromboxane B_2 (TXB_2) concentrations at each sampling time following the oral administration of the racemic drug to the young and elderly volunteers is presented in Figure 3.5. In the young group, the inhibition of TXB_2 generation was greater than 80% from 2.5 to 8 hours post administration, initial peak activity was reached at 3hr with 86% inhibition then there was a slight rise in serum TXB_2 levels before a secondary peak activity of 90% inhibition was observed at 6 hours. In comparison, with the elderly TXB_2 synthesis was inhibited to a level greater than 80% for a longer period, from 0.5 to 10 hours following dosing, with maximum inhibition at 93% achieved after only 2 hours. Furthermore, a residual inhibitory activity of about 25% was still evident in the elderly after 24 hours, whereas with the young TXB_2 concentrations had returned to basal levels by this period. Comparison of the serum concentration versus time profiles of TXB_2 with those of unbound (*S*)-ibuprofen suggest a close relationship (Figure 3.5b). The faster onset and longer-lasting action in the elderly is associated with a shorter T_{max} and slower elimination of unbound (*S*)-ibuprofen in this age group.

The inhibition of adenosine diphosphate (ADP)-induced platelet aggregation was also investigated for serum derived from blood samples collected at 0, 2, 4 and 8 hours post administration (Figure 3.6). However, it appears that ibuprofen's ability to suppress platelet aggregation is not as profound as its inhibitory activity on TXB_2 generation and thus only subtle differences in aggregation were observed between samples times and between the two age groups. However, pairwise comparison of the 2 hr, 4 hr and 8 hr profiles with the initial pre-dose profile (0 hour) indicate optimal inhibition of platelet aggregation after 4 hr and 2 hr in the young and elderly respectively. Furthermore, comparison of the latter time profiles with the two hour sample in the elderly show a slow gradual recovery towards basal aggregation activity; e.g. platelet aggregation induced by 1 μM ADP was 41%, 38% and 25% lower than the basal value for the 2 hr, 4 hr and 8 hr samples respectively.

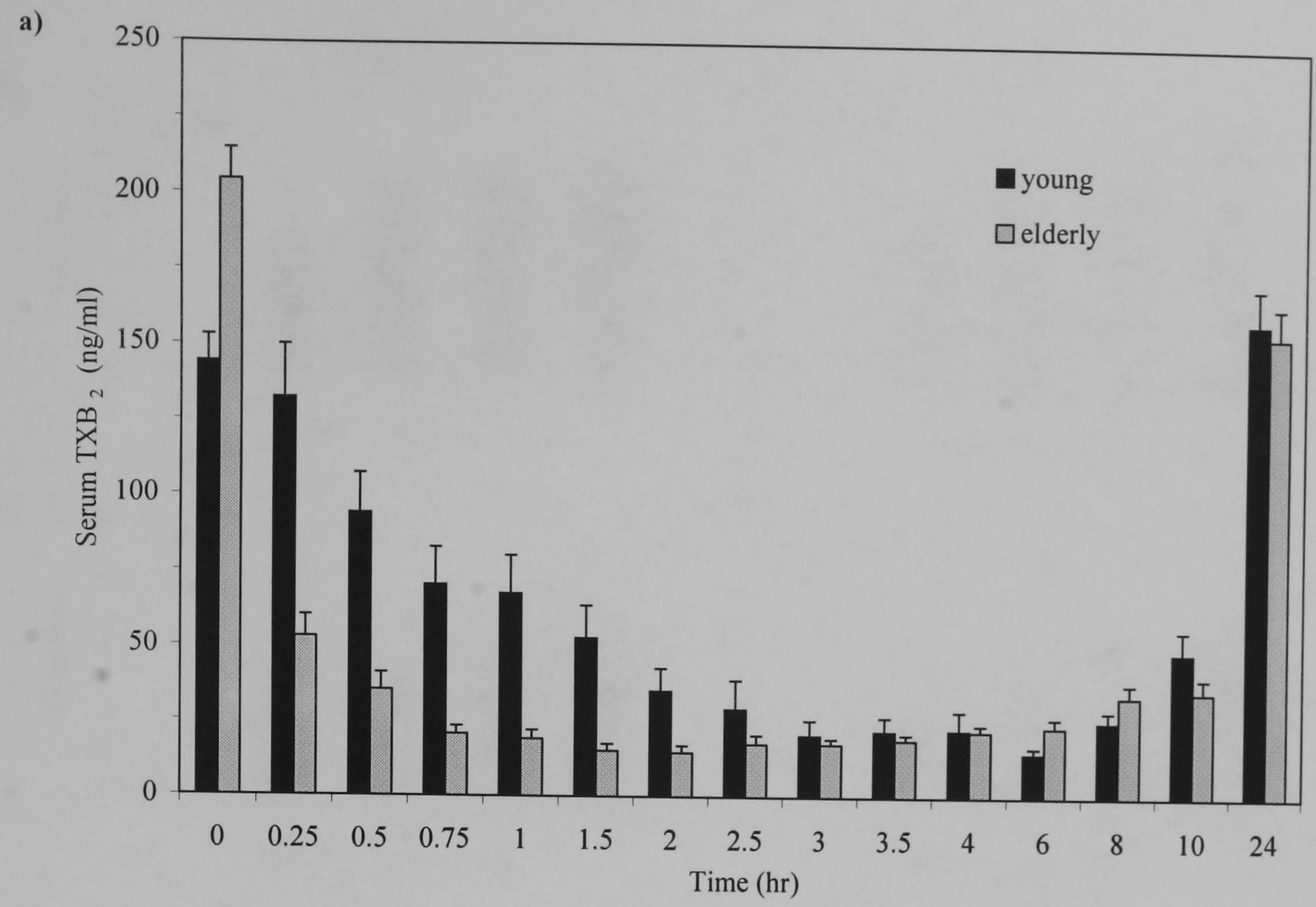
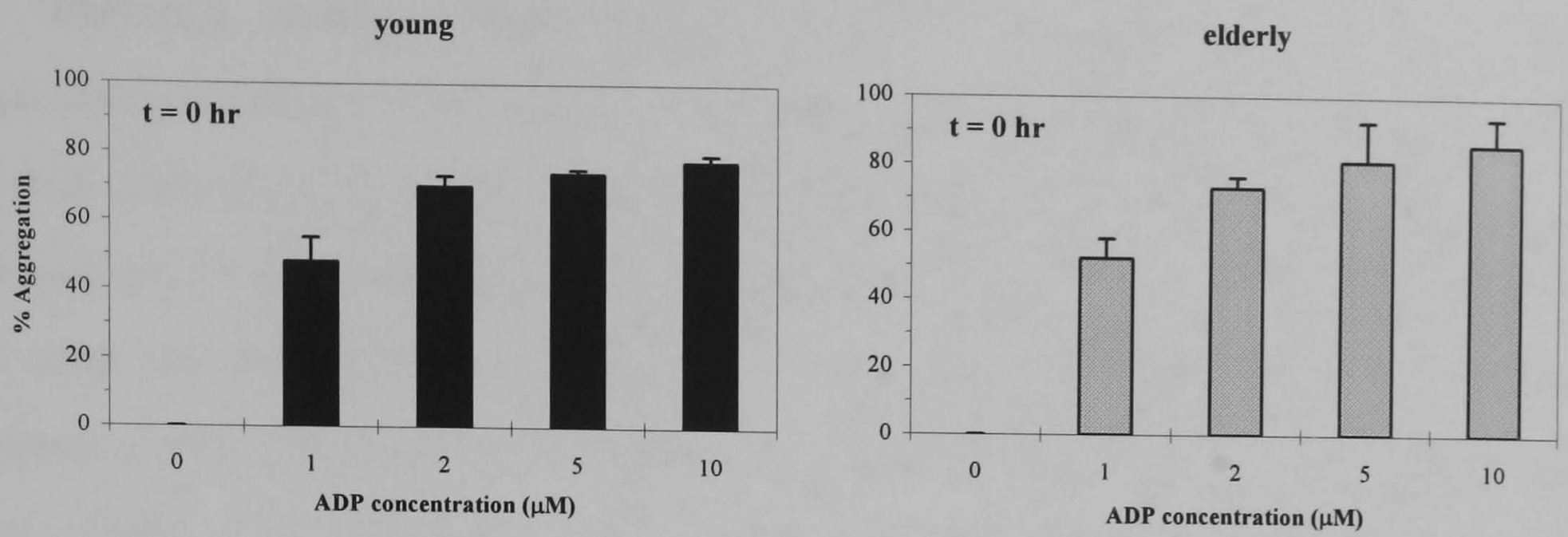
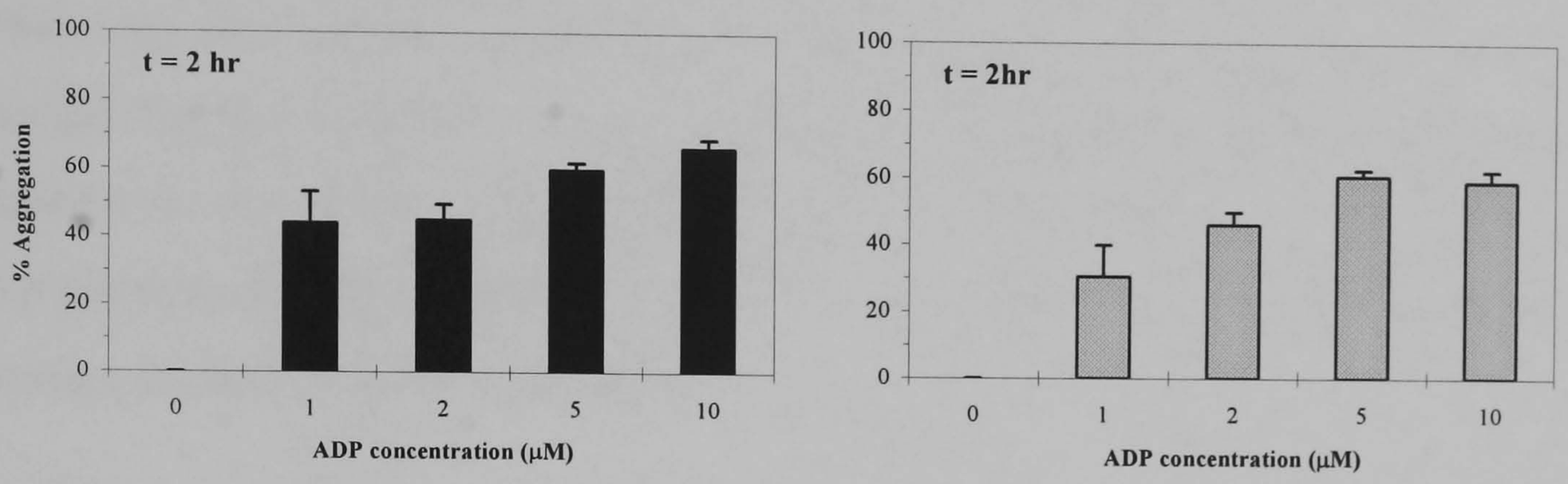


Figure 3.5: (a) *Ex vivo* serum thromboxane B_2 (TXB_2) generation and (b) mean serum concentration versus time profiles of TXB_2 and unbound (*S*)-ibuprofen following the oral administration of racemic ibuprofen (400 mg) to eight healthy young and eight healthy elderly volunteers.

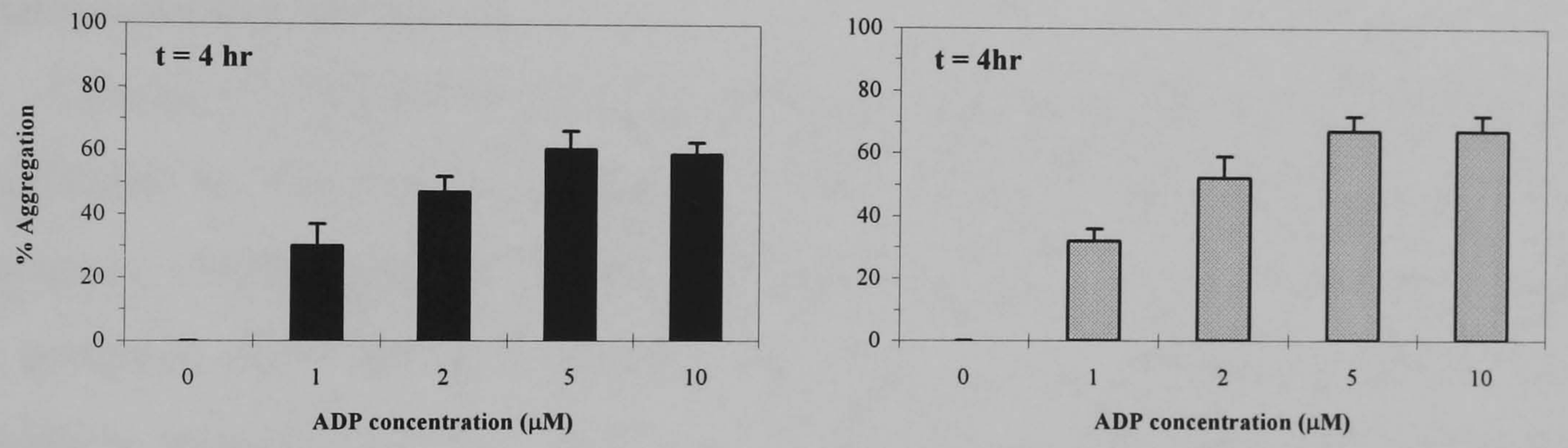
a)



b)



c)



d)

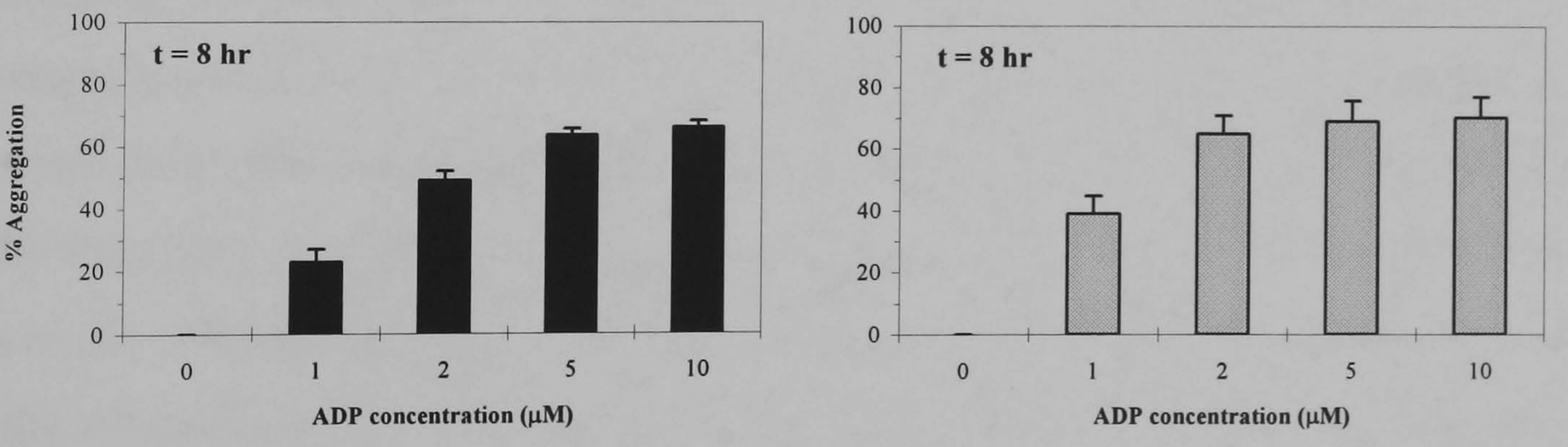


Figure 3.6: ***Ex vivo*** **platelet aggregation induced by adenosine diphosphate (ADP) following the oral administration of racemic ibuprofen (400 mg) to eight healthy young and eight healthy elderly volunteers (mean ± s.d.).**

3.4. Discussion

Previous studies, either non-stereospecific or stereospecific, of ibuprofen disposition in elderly volunteers are rare and have in general suggested that advanced age has only minimal influence on the pharmacokinetics of ibuprofen. However, the conclusions of some of the non-stereospecific investigations may be misleading since, not only do they conceal possible enantiomeric differences, but were based on measuring total (free plus bound) drug concentrations (Greenblatt *et al.*, 1984; Kendall *et al.*, 1990). The importance of unbound serum data is highlighted by Upton *et al.* (1984), who reported that measuring only total concentrations masks an increase in the unbound fraction together with a concomitant decrease in unbound clearance of naproxen in the elderly. The purpose of the current study was to gain a more comprehensive understanding of the effect of age on the enantiomeric disposition and pharmacodynamic activity of ibuprofen.

Enantioselective differences

Clinical investigations into the enantiomeric disposition of ibuprofen in human are complicated by the major role played by the bioactivation of (*R*)-ibuprofen to the *S*-antipode. Pharmacokinetic characterisation of (*S*)-ibuprofen must take into account this inversion phenomenon and thus a knowledge of the fractional inversion (F_{inv}) is absolutely essential. In the current study, the fraction inverted was calculated by determining the stereochemical content of the drug and its metabolites in urine. This approach is the most convenient for clinical studies involving the administration of the racemate since alternative methods, based on serum analysis, require the administration of (*R*)-ibuprofen, a pseudoracemate or the individual enantiomers on separate occasions (Lee *et al.*, 1985; Baillie *et al.*, 1989; Rudy *et al.*, 1991). Implicit in the adopted method are the assumptions that all the dose is ultimately recovered in the urine and that the metabolites do not undergo inversion to an appreciable extent. Almost complete urinary recovery (95%) of an oral administered dose of radiolabelled ibuprofen has been observed (Lee *et al.*, 1985) and thus it is possible that the 25 to 35 % of the dose not recovered and characterised in the current study may have an influence, albeit small, on the determined F_{inv} values. The second assumption is supported by *in vitro* evidence that neither the hydroxy- or carboxy- metabolites inhibit the formation of (*R*)-ibuprofenyl-CoA thioester (Hall *et al.*, 1993; Tracy *et al.*, 1993). The estimates of the extent of

inversion were similar for the two age groups and fall within the range of 0.52 to 0.74 reported in the literature (Lee *et al.*, 1985; Baillie *et al.*, 1989; Hall *et al.*, 1993; Smith *et al.*, 1994; Scheueuer *et al.*, 1998).

In addition to chiral inversion, stereoselectivity in protein binding will also have a major influence on the stereoselective disposition of ibuprofen. The observation of significantly higher unbound fractions of (*S*)-ibuprofen for both age groups is consistent with *in vitro* binding studies that indicate (*R*)-ibuprofen has a 2.3-fold higher binding constant than (*S*)-ibuprofen for the high affinity binding site, namely site II (diazepam binding site), on human serum albumin (Itoh *et al.*, 1997).

In general, most of the enantioselective differences observed in the in pharmacokinetic parameters, i.e. for volume of distribution (V_d), total clearance (CL) and elimination half-life ($t_{1/2,z}$), were evident in both age groups. When unbound serum concentrations of the ibuprofen enantiomers are considered, the enantioselectivity is lost for volume of distribution (V_{du}) and reversed for unbound clearance (CL_u), clearly emphasising the central importance of plasma protein binding. Furthermore, enantioselective differences were observed for unbound area under the curve (AUC_u) in both age groups; however, such enantioselectivity was observed to a lesser degree in the young or not evident at all in the elderly for AUC values based on total serum concentrations.

The role played by stereoselective protein binding in (*S*)-ibuprofen having a significantly higher V_d than its antipode (Tables 3.1 and 3.4) is apparent from the following expression:

$$\mathbf{V_d = V_p.(1 + R_{E/I}) + V_T.(f_u/f_T)} \qquad \text{(Eqn. 3.13)}$$

where V_p is the plasma volume, $R_{E/I}$ is the ratio of plasma binding protein in the extracellular fluid to plasma, V_T is the volume of extravascular tissue and f_u and f_T are the fractions of unbound drug in plasma and tissue respectively. Assigning physiological values to $R_{E/I}$, V_p and V_T of 1.4, 3.0 L and 30 L respectively followed by substitution into equation 3.13 yields (Oie and Tozer, 1979; Lin *et al.*, 1987; Evans, 1992):

$$\mathbf{V_d = 7.2 + 30.(f_u/f_T)} \qquad \text{(Eqn. 3.14)}$$

As tissue binding is of minor importance with ibuprofen, it is therefore clear that the larger V_d of the *S*-enantiomer is a result of the higher unbound fraction in plasma. This is corroborated by the fact that this enantiomeric difference disappears when f_u is not an issue as with the case of V_{du}, i.e. the unbound V_d are not significantly different between the two enantiomers (Tables 3.3 and 3.6).

While the total CL of (*S*)-ibuprofen was significantly greater than that of the *R*-enantiomer, the reverse situation was observed when unbound clearance values were examined, i.e. CL_u *R*>*S*, indicating that the apparent higher total CL for the *S*-enantiomer was due to the higher free serum concentrations. The reversal of the enantioselective preference from CL to CL_u is reflected in AUC_u displaying considerably greater enantioselectivity than evident for AUC, i.e. the mean enantiomeric ratios (*S/R*) for AUC and AUC_u were 1.3 and 2.6 respectively in the young, and 1.1 and 2.7 in the elderly. As expressed above, both the V_d and CL parameters exhibit enantiomeric differences having higher values for the *S*-enantiomer, however the magnitude of the enantioselectivity is greater for V_d such that the apparent half-life of (*S*)-ibuprofen is significantly longer than that of (*R*)-ibuprofen in both age groups (Table 3.1 and 3.4).

The unbound clearance for (*R*)-ibuprofen is predominantly due to clearance by inversion ($CL_{inv,u}$) which is of similar magnitude to the unbound clearance of the *S*-enantiomer and approximately 2.2-fold larger than clearance via alternative, non-inversion, pathways ($CL_{other,u}$). These findings contradict those of Rudy *et al.* (1991) who reported that CL_{inv} and CL_{other} of (*R*)-ibuprofen were of equal significance. However, *in vitro* data using rat hepatocytes (Xiaotao and Hall, 1993) and subsequent human dispositional studies are consistent with our findings that CL_{inv} is the main pathway for clearance of (*R*)-ibuprofen (Hall *et al.*, 1993; Smith *et al.*, 1994; Rudy *et al.*, 1995; Scheueuer *et al.*, 1998).

In addition to the metabolic inversion pathway, the principle pathways of ibuprofen elimination involve the formation of ibuprofen glucuronide and oxidation to yield hydroxyibuprofen and carboxyibuprofen. The formation clearances for these metabolites all displayed stereoselectivity in favour of the *S*-enantiomer, which is consistent with the higher CL for (*S*)-ibuprofen. Unbound formation clearances also showed significant preferences for (*S*)-ibuprofen, although the magnitude of the stereoselectivities were less than the corresponding values for total formation clearance.

Thus, as well as the stereoselective protein binding, inherent enantioselectivity in functional oxidation and conjugation makes a major contribute to the enantiomeric disposition of ibuprofen. The stereoselectivity observed for oxidation is in agreement with *in vitro* studies which have shown that cytochrome P450 2C9 (CYP 2C9), the major enzyme mediating 2'- and 3'-position oxidation of ibuprofen, shows substrate stereoselectivity in favour of (*S*)-ibuprofen. As stated in the introduction, *in vitro* studies have also shown glucuronidation to be stereoselective (El Mouelhi *et al.*, 1987); however, the situation *in vivo* is complicated by the possibility of stereoselective hydrolysis of the unstable acyl glucuronide conjugate and thus the enantioselectivity observed is possibly reflective of a net effect (Faed 1984; Knadler and Hall, 1991). The predominance of elimination products having the *S*-configuration, as a result of chiral inversion and stereoselectivity in both protein binding and metabolism, is reflected in the urinary excretion profiles and is schematically depicted in Figure 3.7 (Tables 3.7 and 3.10).

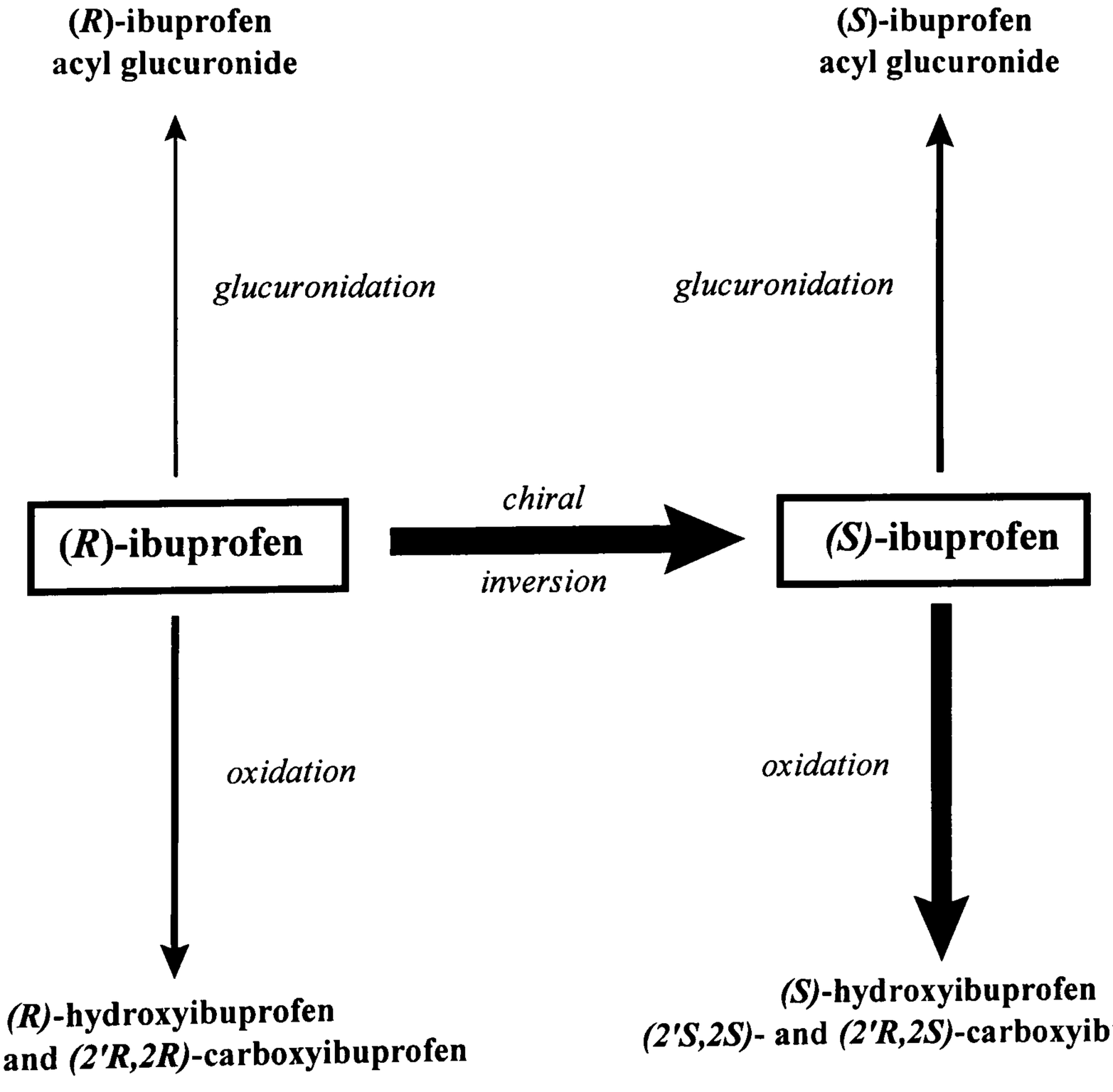


Figure 3.7: Schematic representation of the enantioselective clearance of ibuprofen. The line thickness of the arrows indicate the relative importance of the pathway.

Age-associated differences

Age-associated differences in drug disposition are well established, and age related alterations in enzyme expression may influence the pharmacokinetics of the enantiomers of a racemic drug differently. For example, the oral clearance of (-)-hexobarbitone is about 2-fold greater in young compared with elderly subjects, whereas the clearance of the (+)-enantiomer shows no age-related effects (Chandler *et al.*, 1988). In contrast, the oral clearance of both propranolol enantiomers in young subjects was about 1.5-fold higher than that observed in elderly volunteers, with no effect on the enantiomeric composition of the drug in plasma (Zhou *et al.*, 1992). Age related gender differences have also been observed in enantiomeric disposition (Hooper and Qing, 1990). Examination of the enantiomeric disposition of mephobarbitone in both male and female, young and elderly volunteers indicated the faster elimination of the *R*-enantiomer compared with the *S*-antipode in all groups. However, the oral clearance of (*R*)-mephobarbitone in young males was significantly greater than that observed in either young or elderly females or elderly males, indicating that the clearance of the *R*-enantiomer is both age and gender dependent. No significant differences were evident between young and elderly females or between elderly males and females. In contrast, (*S*)-mephobarbitone has a significantly shorter elimination half life in young males compared with the other volunteer groups, this appears to be as a consequence of differences in distribution since no differences in oral clearance were found between the groups.

Such changes in enantiomeric disposition with age may influence therapeutic response, depending upon whether the eutomer or the distomer is most affected, and may contribute to the observed differences in drug sensitivity, either enhanced pharmacological activity or adverse effects, found in the elderly.

The present investigation has shown that following the oral administration of a single dose of racemic ibuprofen to young and elderly volunteers age-related differences were observed in the disposition of the *S*-enantiomer but not its *R*-antipode. Such changes in the handling of the *S*-enantiomer appear to originate from a 15% greater unbound fraction and a 17% reduction in the unbound clearance in the elderly. In terms of the pharmacokinetics of (*S*)-ibuprofen, this is reflected by a significantly lower C_{max} and longer $t_{1/2,z}$ in the advanced age group; furthermore, there was also a trend towards

the elderly having a greater V_d and, as would be expected, larger AUC_u for this enantiomer.

In the case the *R*-enantiomer, it would have been expected that any prominent age related changes would have most likely stemmed from differences in the extent of chiral inversion between the two age groups, since clearance via this route is *ca*. two fold more important than other metabolic pathways. However, the mean inversion fraction for the two age groups showed good correlation (Tables 3.3 and 3.6). It is also worth noting, that the inter-subject variability in the fractional inversion is relatively small, indicating that the chiral inversion appears to be a relatively "stable" process, i.e. the fraction of the dose presented to the body in the active form is relatively predictable in healthy individuals. Other major influential factors, such as protein binding and total unbound clearance, also displayed no age associated effect and therefore it is not surprising that age-dependent alterations in the disposition of (*R*)-ibuprofen are not apparent.

This study indicates that ageing stereoselectively increased the unbound fraction of (*S*)-ibuprofen without any alteration in the binding of the *R*-antipode. Whilst it is generally accepted that increases in unbound fraction may be due to lower serum albumin concentrations in the elderly (Wallace and Verbeeck, 1987; Grandison and Boudinot, 2000), this appears not to be applicable in the present study since the determined serum albumin concentrations for the two volunteer groups were not significantly different (see Appendix 1). This is similar to the observations of Upton *et al.* (1984), where a small difference in serum albumin levels between the young and elderly volunteers could not account for the larger difference in plasma protein binding of naproxen. Furthermore, any general reduction in albumin concentrations would have expected to cause a parallel decrease in the binding of (*R*)-ibuprofen. It has also been suggested that the increase in unbound (*S*)-ibuprofen in the elderly may be due to accumulated oxidative metabolites acting as selective displacers (Rudy *et al.*, 1995). However, it is unlikely this is the case since the unbound formation clearances of the metabolites tended to be lower in the elderly.

Examination of the unbound formation clearances suggest that the trend towards a lower unbound clearance of (*S*)-ibuprofen in the elderly is largely due to a reduction in oxidative metabolic function. Recent *in vitro* investigations have suggested that CYP 2C8 may also play a significant role alongside the principle metabolising enzyme,

CYP2C9, in the oxidative metabolism of ibuprofen (Hamman *et al.*, 1997). It is interest to note that the CYP2C8 displays opposing enantioselectivity to CYP 2C9 and favours the formation of products with the *R*-configuration. It is likely that age has a relatively lower influence on CYP 2C8 than CYP 2C9-mediated metabolism, explaining why the clearance of (*R*)-ibuprofen via the oxidative pathways was altered to a lesser extent between the two age groups. Clearance via the minor glucuronidation pathway was comparatively indifferent between the young and elderly, which is agreement with previous suggestions that glucuronidation is generally not affected by ageing (Mooney *et al.*, 1985).

However, as the changes in protein binding and unbound clearance appear to operate in opposite directions no apparent age-related difference is observed in the total clearance of (*S*)-ibuprofen, i.e. the increase in fraction unbound with age (young, 0.46% and elderly, 0.53%) negates the observed decrease in unbound clearance (young, 16.0 L/min and elderly, 13.3 L/min). Thus, at steady state the total serum concentrations of (*S*)-ibuprofen would appear to be similar between the two groups, but this would mask the fact that the elderly have exposure to higher unbound concentrations. This can best be appreciated by the observation that the total AUC was similar between the two age groups (young, 78.5 mcg/ml hr and elderly 84.0 mcg/ml hr), whereas the unbound AUC was 25% greater in the elderly (young, 0.36 mg/ml hr and elderly 0.45 mg/ml hr). The increase in the unbound fraction with age is paralleled with a trend towards a higher volume of distribution and this results in the elderly exhibiting a significantly longer half life for (*S*)-ibuprofen than the young group.

The clinical implications of the stereoselective increase in the exposure of the elderly to the free levels of the active enantiomer of ibuprofen can not be fully appreciated solely on pharmacokinetic knowledge. Aside from the concentration of the drug at its site of action, the magnitude of a drug effect depends on the number of receptors in the target organ, the capability of the cells to respond to specific occupation of the receptors (signal transduction) and the counterregulatory processes operating in the body. Hence, in addition to pharmacokinetics, drug pharmacodynamics may be changed in the elderly (Hammerlein *et al.*, 1998). For example, both animal and human studies suggest that there is an inverse relationship between advancing age and gastrointestinal prostaglandin levels and thus it has been postulated that NSAIDs cause

more gastropathy in older individuals due to lower basal levels of prostaglandins (Solomon and Gurwitz, 1997).

To monitor the pharmacodynamic activity of ibuprofen on platelet cyclooygenase, the amount of thromboxane A_2 (TXA_2) generated in response to endogenous thrombin formation (i.e clot formation) was assessed by measuring the concentration of its stable breakdown product, thromboxane B_2 (TXB_2), in the harvested serum (Figure 3.5). The potent activity of ibuprofen is clearly evident with near complete inhibition of serum TXB_2 (>90 %) in both age groups, which is in good agreement with the extent of inhibition observed by Evans *et al.* (1991). The close transitory relationship between inhibition of TXB_2 generation and serum unbound (*S*)-ibuprofen concentration in both the young and elderly groups is consistent with the close proximity of the biological receptor (platelet cyclooxygenase) to the sampling compartment (serum). The time course of inhibition is in keeping with the fact that the binding of ibuprofen to platelet cyclooxygenase is a reversible process (Cashman, 1996). In contrast, the function of platelets which have been exposed to aspirin cannot be restored, since this drug irreversibly acetylates the active site on the cyclooxygenase (Flower *et al.*, 1985). Comparison of the TXB_2 profiles for the young and elderly groups mirror the differences evident in the unbound (*S*)-ibuprofen kinetic profiles and thus suggest that inhibitory activity has faster onset and is longer-lived in the elderly. It is interesting to note that the serum TXB_2 concentration was 25 % below the pre-treatment value at 24 hr in the elderly. Whereas with the young, TXB_2 concentrations had returned to basal activity as has been observed previously for four healthy young males (Evans *et al.*, 1991). The maintenance of such a significant degree of activity in the elderly at 24 hour post dosing is unlikely to be solely related to differences in serum concentrations of unbound (*S*)-ibuprofen. The elderly have an increased proportion of body fat (Dawling and Crome, 1989) so it is possible that the prolonged activity could be associated with the leaching of (*S*)-ibuprofen from adipose stores (Evans, 1991). Alternatively, it could be reflective of the fact that the elderly are more sensitivity to the effects of ibuprofen, this could be investigated through pharmacokinetic-pharmacodynamic modelling, however this is beyond the scope of the current investigation.

Platelet aggregation studies were also performed using platelet rich plasma and adenosine diphosphate (ADP) as an external stimulus for aggregation (Figure 3.6). Age related differences were more difficult to define, however there was a trend consistent with the findings of the TXA_2 study suggesting that activity was observed faster and for

a longer period in the elderly group. The main disadvantage of the platelet aggregation studies is that they are time-consuming and need to be performed using freshly isolated plasma from recently drawn blood, since they are dependent on the viability of the arachidonic acid pathway in the platelets, and so only a restricted number of sampling times can be assessed.

3.5. Conclusions

Similar stereoselective differences were observed in the pharmacokinetics of the enantiomers of ibuprofen following the administration of the racemic drug to healthy young and elderly volunteers. Stereoselective protein binding favouring higher serum unbound (*S*)-ibuprofen concentrations led to enantiomeric differences in V_d and the unbound AUC. (*R*)-Ibuprofen was cleared predominately through chiral inversion and clearance through the oxidation and glucuronidation metabolic pathways, exhibited substrate stereoselectivity in favour of the *S*-enantiomer. Metabolite formation clearances indicate that the stereoselectivity in these metabolic pathways is as a result, not only of the stereoselective protein binding, but also inherent stereoselectivity in the metabolic reactions.

Age-related alterations were only evident in the disposition of (*S*)- and not (*R*)-ibuprofen. As a consequence of a higher unbound fraction and reduced unbound clearance, which was largely due to reduced clearance via oxidative pathways, the elderly had a greater exposure to the free levels of the active (*S*)-ibuprofen. Pharmacodynamic studies, based on monitoring the inhibition of platelet TXA_2 generation, shows that activity is in close correlation with serum concentrations of unbound (*S*)-ibuprofen. This would suggest that age-related changes in the disposition of (*S*)-ibuprofen could have significant clinical implications and necessitate dose reduction in the elderly.

CHAPTER 4 :

Chromatographic separation and enantiomeric resolution of flurbiprofen and its metabolites

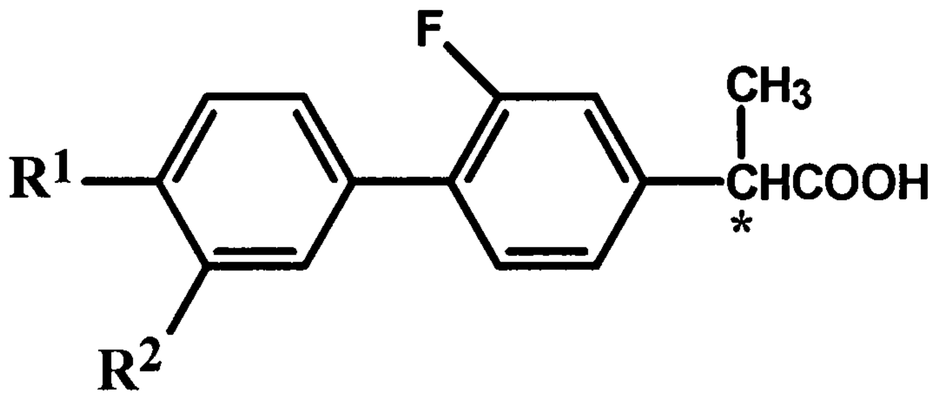
4.1. Introduction

Since the first reported enantiospecific analytical methods became available in the early 1970s, a wide range of methods and techniques have been developed for the enantiospecific analysis of chiral NSAIDs. The indirect chromatographic approach, i.e. involving the formation of stable diastereomeric derivatives is the more popular technique in terms of validated assays published in the literature (Davies, 1997). The number of commercially available homo-chiral derivatizing reagents (HCDAs), many of which are available in very high and reliable enantiomeric purity, has increased in recent years and several compilations of both reagents and derivatization methods have been published (Hutt, 1990; Gal, 1993; Skidmore, 1993; Görög and Gazdag, 1994; Büschges *et al.*, 1997). Direct methods, utilising chiral stationary phases (CSPs) or chiral mobile phase additives (CMPAs), are still relatively new and their application in bioanalysis is more limited. But their potential is certainly evident and they are becoming increasing employed as novel phases and modifications to existing phases, with improvements in both selectivity and versatility, continue to be introduced (Major, 1999; Terfloth, 1999).

It is therefore the more surprising that there remains a lack of published enantiospecific assays for flurbiprofen, one of the most popular and widely used 2-aryl-propionic acid drugs. Indirect methods for the determination of flurbiprofen enantiomers have been based on the formation of diastereomeric amides after derivatization with optically pure chiral amines such as (*S*)-1-phenylethylamine (Maitre *et al.*, 1984; Adams *et al.*, 1988; Knadler and Hall, 1989) and L-leucinamide (Berry and Jamali, 1988). The direct approach, utilising the protein CSPs, α_1-acid glycoprotein (Geisslinger *et al.*, 1992) and ovomucoid (Fukuhara *et al.*,1996), have been employed for the determination of flurbiprofen in plasma; and the resolution of the methyl esters of flurbiprofen was achieved using a cellulose tris (4-methylbenzoate) phase by Aboul-Enein and Bakr (1992). Recently, a sequential achiral-chiral method has been used for the enantiomeric quantification of flurbiprofen in rat plasma based on pre-column derivatization with the nonchiral fluorogenic reagents, 4-[*N,N*-dimethylamino)sulphonyl]-7-piperazino-2,1,3-benzoxadiazole or 4-[[*N*-hydrazinoformyl)methyl]-*N*-methyl]amino-7-[*N,N*-dimethyl-amino)sulphonyl]-2,1,3-benzoxadiazole and chiral resolution carried out using a cellulose tris (3,5-dimethyphenylcarbamate) CSP (Fukushima *et al.*, 1997).

The major metabolites of flurbiprofen, namely 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen, retain the asymmetric centre and therefore may also

exist in the *R*- and *S*-configurations (Figure 4.1). Of all the assays outlined above, only one methodology considers the quantification of the individual enantiomers of the metabolites as well as those of the parent drug (Knadler and Hall, 1989). However, due to their different polarities, flurbiprofen and its metabolites could not be analysed using a single isocratic system on a achiral C_{18} column and changes in the mobile phase were required. Furthermore, the quantification of flurbiprofen in urine required both fluorescence and UV detection since the internal standard had negligible fluorescent activity. Due to the lack of authentic standards of the individual enantiomers of the metabolites the stereochemical elution orders were defined by analysing urine obtained from a rat dosed with (*S*)-flurbiprofen.



	R^1	R^2
Flurbiprofen	**H**	**H**
4'-Hydroxyflurbiprofen	**HO**	**H**
3'-Hydroxy-4'-methoxyflurbiprofen	**CH_3O**	**HO**

Figure 4.1: Chemical structures of flurbiprofen and its major metabolites (* denotes chiral centre).

It is clear from the above that there is a need for a simple chromatographic separation method which is able to resolve the enantiomers of flurbiprofen and its metabolites in a single chromatographic run and which can be routinely employed in bioanalytical studies. In the present study a variety of chromatographic methods were investigated for their suitability : (a) indirect separation on an achiral reversed-phase system following the formation of diastereomeric derivatives using (*R*)-1-(naphthen-1-yl)ethylamine, (b) direct separation using hydroxypropyl-β-cyclodextrin as a CMPA in a reversed-phase HPLC system, (c) direct separation on a α_1-acid glycoprotein CSP via reversed-phase HPLC and (d) direct separation on a amylose tris (3,5-dimethyl-phenylcarbamate) CSP via normal-phase HPLC. These various approaches present

different mechanisms for chiral discrimination and the advantages and limitations of each will be outlined.

The technique which displayed optimal separation characteristics was selected for further evaluation for it clinical applicability, this is presented in Chapter 5. Furthermore, this method with modifications in mobile phase composition was used for the semi-preparative isolation of the individual enantiomers of the metabolites for stereochemical assignment using circular dichroism (CD).

4.2. Experimental

4.2.1 Chemicals and reagents

Acetonitrile, dichloromethane, ethanol, hexane, isopropanol and methanol (HPLC grade) were purchased from Rathburn (Walkerburn, UK). Trifluoroacetic acid (TFA) and sodium hydrogen phosphate (GPR grade) were purchased from BDH (Poole, Dorset, UK). *N,N*-Dimethyloctylamine (DMOA) was obtained from Aldrich Chemicals (Gillingham, Dorset, UK), 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride (CDI) and (*R*)-1-(naphthen-1-yl)ethylamine ((*R*)-NEA) were purchased from Sigma Chemicals (Poole, Dorset, UK). 1-Hydroxybenzotriazole (HOBT) was purchased from Fluka Chemicals (Poole, Dorset, UK). Hydroxypropyl-β-cyclodextrin preparations: HP-β-CD-**I** (Molar substitution = 0.6, average MW = 1380) from Aldrich Chemicals (Gillingham, Dorset, UK); HP-β-CD-**II** (pharmaceutical grade) and HP-β-CD-**III** (standard grade) were the generous gifts of Wacker Chemicals Ltd (Egham, Surrey, UK). (*R,S*)-, (*R*)-, (*S*)-Flurbiprofen, (*R,S*)-4'-hydroxyflurbiprofen and (*R,S*)-3'-hydroxy-4'-methoxyflurbiprofen were the generous gifts of Boots Company PLC (Nottingham, UK)

4.2.2 Chromatographic columns

Waters Resolve C_{18} column (150 x 3.9 mm, 5 μm) was obtained from Anachem Ltd. (Luton, Beds., UK), and used with a refillable guard column (10 x 2.1 mm) packed with pellicular C_{18} (40-60 μm) both were obtained from Alltech Associates (Carnforth, Lancs, UK). Waters Symmetry C_{18} column (75 x 4.6 mm, 3.5 μm) and Waters Sentry

Guard, fitted with a Symmetry C_{18} cartridge (10 x 4.6 mm, 3.5 μm), were purchased from Phase Separations Ltd. (Deeside, Flints., UK). The Chiral-AGP (α_1-acid glycoprotein) column (100 x 4.0 mm, 5 μm) with a guard column (10 x 3.0 mm, 5 μm) filled with the same material, both were obtained from ChromTech AB (Norsborg, Sweden). The Chiralpak AD (amylose tris (3,5-dimethylphenylcarbamate)) column (250 x 4.6 mm, 10 μm) used with a matching guard column (50 x 4.6 mm, 10 μm) was supplied by HPLC Technology Ltd. (Macclesfield, UK).

4.2.3 Instrumentation

Reversed-phase HPLC was performed using an LDC Constametric 3000 pump linked to an LDC Spectromonitor 3000 UV detector and a LDC CI-4000 computing integrator (Stone, Staffs., UK). Samples were introduced on-column using a Rheodyne 7125 injection valve (Cotati, Cal., USA) fitted with a 20 μl sample loop. When fluorescence detection was required a Kontron SFM 25 fluorescence detector (Watford, Herts., UK) was connected in series, prior to, the UV detector.

HPLC utilising the Chiralpak AD CSP was performed using an LDC Constametric 3000 pump linked to an LDC Spectromonitor 3100 UV detector and a LDC CI-4100 computing integrator (Stone, Staffs., UK). Samples were injected on column using a Perkin Elmer ISS-100 autosampler (Beaconsfield, Bucks., UK).

Circular dichroism (CD) spectra were recorded using a Jasco J600 spectropolarimeter (Halstead, Essex, UK).

4.2.4 Development of a chromatographic methods for the resolution of the enantiomers of flurbiprofen and its major metabolites

Various enantiospecific chromatographic approaches were investigated for the resolution of the enantiomers of flurbiprofen , 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen.

Method 1 : Chromatographic separation after pre-column derivatization

The derivatization procedure is similar to that described for ibuprofen in section 2.2.4, which utilised flurbiprofen as an internal standard. Standard solutions of

flurbiprofen, 4'-hydroxyflurbiprofen or 3'-hydroxy-4'-methoxyflurbiprofen were prepared in dichloromethane (1mg/10ml). To 10 µl aliquots of the standard solutions were added 100 µg each of CDI, HOBT and (*R*)-NEA (100 µl each of a 1mg/ml solution in dichloromethane). The tubes were tightly capped and the derivatization procedure performed as before.

Chromatography was carried out using a Waters Resolve C_{18} (150 x 3.9 mm, 5 µm) column protected by a guard column (10 x 2.1 mm) filled with pellicular C_{18}. The mobile phase used was phosphate buffer (pH 3.5, 0.01M) : acetonitrile (50:50 v/v) at a flow rate of 0.8 ml/min. Column eluate was monitored by UV detection at 254 nm or by fluorescence detection with excitation and emission wavelengths of 290 and 330 nm respectively. Derivatized standards were reconstituted in 50 µl mobile phase and 20 µl aliquots injected into the HPLC.

Method 2 : Direct chromatographic separation using a CMPA

Chromatography was performed using a Waters Symmetry C_{18} column (75 x 4.6 mm, 3.5 µm) protected by a Waters Sentry Guard fitted with a Symmetry C_{18} cartridge (10 x 4.6 mm, 3.5 µm). The mobile phase consisted of aqueous TFA (pH 3.5; 0.1% v/v) : methanol (60:40 v/v) containing HP-β-CD (1.1 % w/v), at a flow rate of 1.0 ml/min at ambient temperature. Detection was by UV absorbance at 254 nm. Samples were injected onto the system as 20 µl of 1mg/10ml solutions in mobile phase.

Method 3 : Direct chromatographic separation using a Chiral-AGP CSP

Chromatography was carried out using a Chiral-AGP column (100 x 4.0 mm, 5µm) linked to a matching guard column (10 x 3.0 mm, 5 µm). The mobile phase consisted of phosphate buffer (pH 6.5, 0.01 M):isopropanol (98.5:1.5 v/v) containing DMOA (1.0 mM). The flow rate was set at 0.5ml/min and detection was by UV absorbance at 254 nm. The system was used in an air-conditioned room at 23°C. Standard solutions of flurbiprofen and its metabolites (1 mg/10ml) were prepared in mobile phase and 20 µl aliquots where subjected to analysis.

Method 4 : Direct chromatographic separation using a Chiralpak AD CSP

Chromatography was carried out using a Chiralpak AD column (250 x 4.6 mm, 10 µm) linked to a matching guard column (50 x 4.6 mm, 10 µm). The mobile phase

consisted of hexane:ethanol (90:10 v/v) containing trifluoroacetic acid (0.05% v/v) as modifier, run at a flow rate of 1.0ml/min. Detection was by UV absorbance at 254 nm. Standard solutions of flurbiprofen and its metabolites (1 mg/10ml) were prepared in mobile phase and 20 µl aliquots where subjected to analysis.

Characterisation of chromatographic separations

In order to evaluate the chromatographic performance of the various approaches in the enantioseparation of flurbiprofen and its metabolites, several factors were investigated. The retention factors (k'), the separation factor (α) and the resolution value (R_S) were calculated for each analyte using the following equations:

$$k' = (t_r - t_o) / t_o \qquad \text{(Eqn. 4.1)}$$

where t_r is the retention time of the enantiomer and t_o is the column dead time determined by the injection peak;

$$\alpha = k'_2 / k'_1 \qquad \text{(Eqn. 4.2)}$$

where k'_1 and k'_2 are the capacity factors of the first and second eluting enantiomers respectively.

$$R_S = 2\,(t_2 - t_1) / (w_1 + w_2) \qquad \text{(Eqn. 4.3)}$$

where t_1 and t_2 are the retention times of the first and second enantiomers respectively and w_1 and w_2 are the peak widths at the base of the first and second enantiomers respectively. R_S is a measure of the extent of overlap of the two peaks and ideally should be greater than 1.5 (indicating baseline resolution) if the chromatographic separation is to be used for quantitative analysis (Gal, 1993).

4.2.5 Semi-preparative chromatographic resolution of the enantiomers of 4'-hydroxyflurbiprofen

In order to obtain sufficient quantities of each 4'-hydroxyflurbiprofen enantiomer for analyte characterisation, repeated injections (50 x 20 µl) of a concentrated analyte solution (20 mg/ml) in ethanol were made onto the Chiralpak AD CSP. The mobile

phase used was hexane:ethanol (87:13 v/v) containing 0.05% v/v trifluoroacetic acid, at a flow rate of 1.0 ml/min. The eluate was monitored by UV detection at 254 nm. The first eluting peak was collected between 8.9 and 10.9 min and the second between 12.5 and 15.5 min. The mobile phase was evaporated under a stream of nitrogen and the enantiomeric purities of the individual enantiomers then determined by re-examination of an aliquot of each using the CSP.

4.2.6 Semi-preparative chromatographic resolution of the enantiomers of 3'-hydroxy-4'-methoxyflurbiprofen

Repeated injections (50 x 20 μl) of a concentrated 3'-hydroxy-4'-methoxy-flurbiprofen solution (20 mg/ml) in ethanol were made onto the Chiralpak AD CSP. The mobile phase used for separation of the enantiomers was hexane:ethanol (85:15 v/v) containing 0.05% v/v trifluoroacetic acid, run at a flow rate of 1.0 ml/min. The eluate was monitored by UV detection at 254 nm. The first eluting peak was collected between 16.0 and 18.0 min and the second between 22.0 and 25.0 min. The mobile phase was evaporated under a stream of nitrogen and the enantiomeric purities of the individual enantiomers confirmed by re-injection.

4.2.7 Chiroptical characterization of 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen

Solutions in acetonitrile (enantiomers of flurbiprofen and 4′-hydroxyflurbiprofen) and in methanol (enantiomers of flurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen) were prepared at analyte concentration of 0.01mg/ml. Circular dichroism (CD) spectra were recorded over the range 200-320 nm in a cell of pathlength 1 cm at 25°C. In each case a solvent baseline was recorded and subtracted from the analyte spectrum before converting to molar ellipticity (Δε). UV spectra were recorded using the same instrument over the corresponding wavelength range.

4.3. Results and Discussion

4.3.1 Derivatization method

For chiral arylpropionic non-steroidal anti-inflammatory drugs, the presence of a reactive carboxylic acid moiety allows for facile pre-column derivatization with chiral derivatization reagents and this technique has enjoyed extensive application. Chiral amine reagents have been used more frequently than chiral alcohols as reagents since the chromatographic behaviour of diastereomeric amides is often superior to that of respective esters and perhaps because of the greater stability of amides over esters. The majority of amines utilised for derivatization reactions have been 1-substituted ethylamines with a bulky residue such as phenyl, naphthyl or dimethylaminonaphthyl since these agents have the asymmetric centre directly bonded with both the bulky moiety and the reactive amine function. The resulting diastereomeric amides have the ideal properties for optimal separation since the distance between the chiral centres is limited to two atoms and the presence of the bulky substituent encourages conformational rigidity (Görög and Gazdag, 1994).

The enantiomers of 1-(naphthen-1-yl)ethylamine (NEA) are readily available commercially with high optical purity and because of their fluorescent properties have been widely used in biological investigations. (*S*)-NEA was used by Hutt *et al.* (1986) in the derivatization of 2-phenylpropionic acid, ibuprofen, carprofen and pirprofen, and in the analysis of 2-phenylpropionic acid in urine. All derivatives were baseline resolved on a silica-gel radial compression column with hexane-ethyl acetate as a mobile phase. Derivatization with (*S*)-NEA was used for enantiospecific pharmacokinetic studies following the repeated oral administration of ibuprofen (Avgerinos and Hutt, 1987). Subsequently a number of other plasma assay methods based on the formation of the (*S*)-1-(naphthen-1-yl)ethylamide diastereoisomers of ibuprofen have been published (Mehvar *et al.*, 1988; Lemko *et al.*, 1993; Ahn *et al.*, 1994 and Lau, 1996). The derivatives of flurbiprofen and ketoprofen could in addition be conveniently resolved using the method of Mehvar *et al.* (1988), however as yet the assay has not been applied for these profens. The determination of the enantiomers of suprofen in plasma by capillary gas chromatography-mass spectrometry was also based on the pre-column derivatization with this agent (Shinohara *et al.*, 1990). The antipode (*R*)-NEA was employed in the stereospecific determination of the active metabolite of loxoprofen in plasma of humans and dogs (Takasaki and Tanaka, 1992). Recently, Tan *et al.*, (1997a)

published a sensitive enantiospecific assay for the determination of ibuprofen in serum based on the formation of (*R*)-1-(naphthen-1-yl)ethylamide diastereoisomers, which enabled the methodology to be adapted for the determination of the free enantiomer concentrations in serum following equilibrium dialysis.

Activation of the carboxyl groups in flurbiprofen and its metabolites prior to reaction with (*R*)-NEA was essentially the coupling reaction adopted by Tan *et al.* (1997a). The activation agent employed was the carbodiimide, 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide (CDI) in the presence of 1-hydroxybenzotriazole (HOBT), agents originally reported for their use in peptide synthesis. The addition of HOBT is effective not only in suppressing the formation of N-acylisourea by-products but also in enhancing the reaction rates and yields, which is advantageous for the prevention of racemisation (Windridge and Jorgensen, 1971; Benoiton and Kuroda, 1981). The derivatization reaction (Figure 4.2) was allowed to proceed at ambient temperatures for a period of two hours, which is the optimised conditions used previous for the ibuprofen assay methods (see Chapter 2).

Previously, the (*R*)-1-(naphthen-1-yl)ethylamide diastereoisomers of both ibuprofen and flurbiprofen, which had been used as an internal standard, were successfully resolved using a reversed-phase HPLC system (see Chapter 2). Using the same stationary phase, Waters Resolve C_{18} column, the system was investigated by alteration of the mobile-phase composition and/or flow rate to determine if the diastereoisomeric derivatives of flurbiprofen and its major metabolites, 4'-hydroxy-flurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen, could all be separated in a single chromatographic run. The chromatogram obtained using a mobile phase of phosphate buffer (pH 3.5, 0.01M):acetonitrile (50:50 v/v) at a flow rate of 0.8 ml/min using UV detection is shown in Figure 4.3a. Each pair of analyte derivatives showed baseline separation, as indicated by R_S values > 1.5 (Table 4.1). However, (*R*)-3'-hydroxy-4'-methoxyflurbiprofen was not completely resolved from (*S*)-4′-hydroxyflurbiprofen (Figure 4.3a). Decreasing the flow rate further, or reducing the proportion of organic modifier to complete separation would probably result in run times unsuitable for routine analysis.

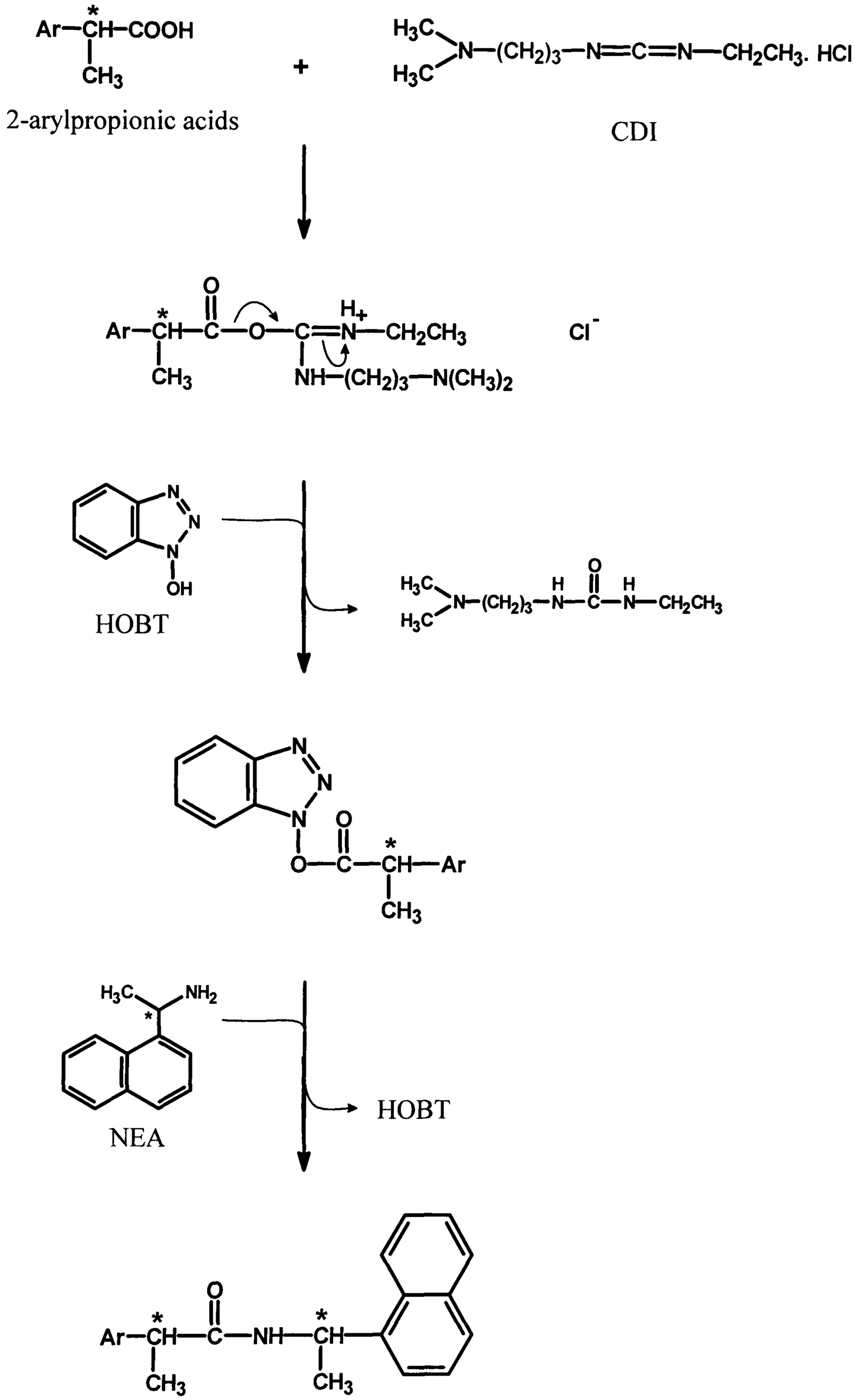


Figure 4.2: Derivatization of 2-arylpropionic acids with 1-(naphthen-1-yl)ethylamine (NEA) in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (CDI) and 1-hydroxybenzotriazole (HOBT).

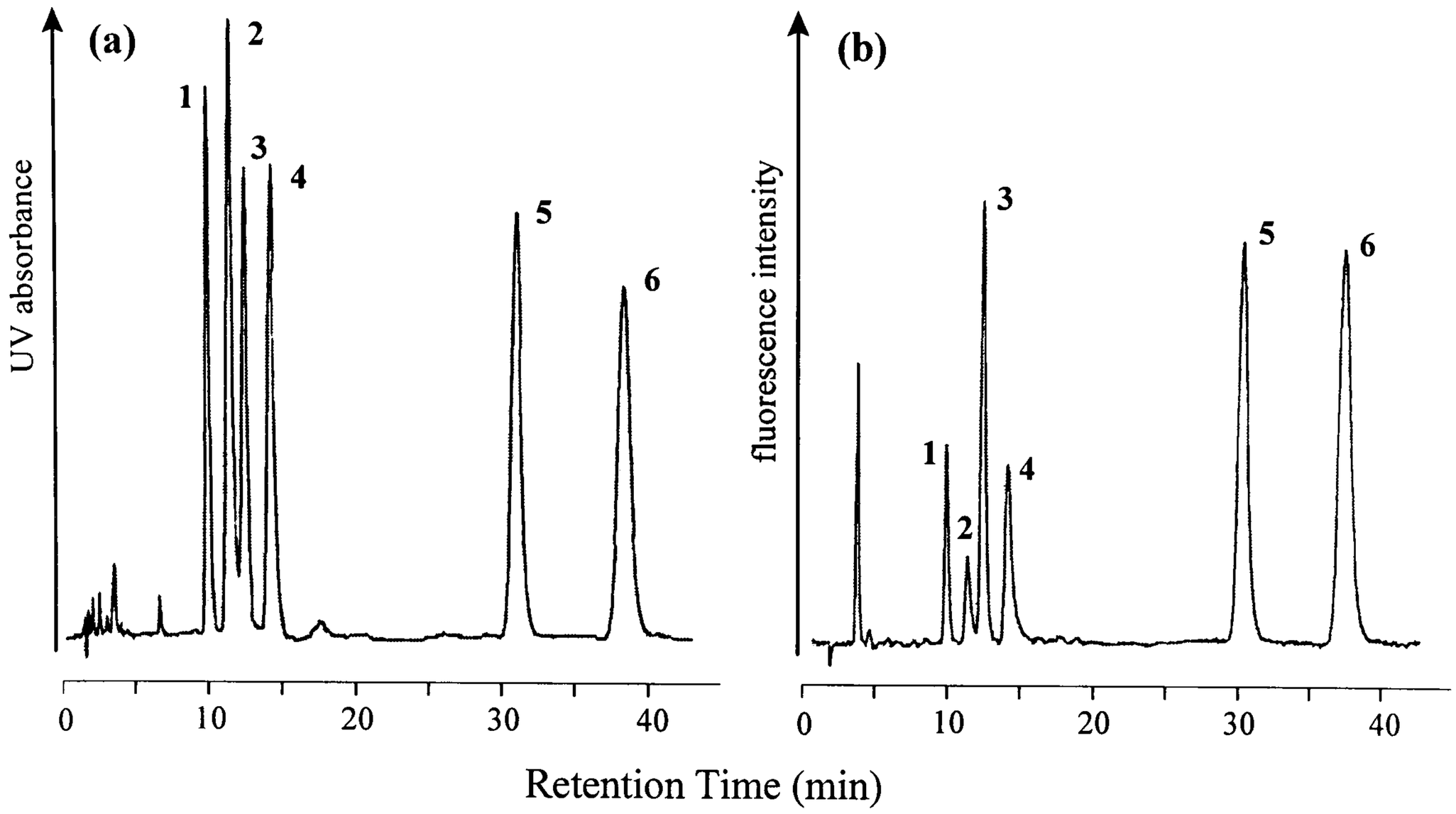


Figure 4.3: Chromatograms of flurbiprofen and its major metabolites following derivatization with (*R*)-NEA using (a) UV detection and (b) fluorescence detection. Peaks : (*R*)-1-(naphthen-1-yl) ethylamide derivatives of 1, (*R*)-4'-hydroxyflurbiprofen; 2, (*R*)-3'-hydroxy-4'-methoxyflurbiprofen; 3, (*S*)-4'-hydroxyflurbiprofen; 4, (*S*)-3'-hydroxy-4'-methoxyflurbiprofen; 5, (*R*)-flurbiprofen and 6, (*S*)-flurbiprofen. [Stationary phase, Resolve C_{18} column (150 x 3.9 mm, 5 μm); Mobile phase, phosphate buffer (pH 3.5, 0.01M):acetonitrile (50:50 v/v); Flow rate, 0.8 ml/min]

Table 4.1: Chromatographic parameters for the separation of the (*R*)-1-(naphthen-1-yl) ethylamide diastereoisomers of flurbiprofen and its metabolites on a Waters Resolve C_{18} column *.

Analyte	k'_1	k'_2	α	R_S
Flurbiprofen	10.76	13.58	1.26	2.76
4'-Hydroxyflurbiprofen	2.69	3.68	1.37	2.19
3'-Hydroxy-4'-methoxyflurbiprofen	3.27	4.37	1.34	2.00

** Mobile phase : phosphate buffer (pH 3.5, 0.01M):acetonitrile (50:50 v/v.)*
Elution order : R- before S-enantiomer for all three analytes.

The fluorescent properties of the (*R*)-1-(naphthen-1-yl)ethylamide derivatives are advantageous for increasing the sensitivity and selectivity of the approach for use in the analysis of biological fluids. Figure 4.3b shows a typical chromatogram obtained when the column eluate was monitored by a fluorescence detector (λex: 290nm and λem: 330nm) connected in series, prior to the UV detector. For all three analytes it was

observed that the two diastereomeric derivatives exhibited unequal fluorescence intensities. The *R/S* peak-area ratios for the derivatives of flurbiprofen, 4'-hydroxy-flurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen were 0.82, 0.43 and 0.35 respectively. Furthermore, adjustment of the fluorescence condition to those previously employed by Knadler and Hall (1989) to monitor the (*S*)-1-phenylethylamides of the metabolites of flurbiprofen, i.e. with excitation and emission wavelengths of 260 and 320 nm respectively, also showed unequal fluorescent activity between diastereomeric derivatives; with *R/S* peak-area ratios for the derivatives of flurbiprofen, 4'-hydroxy-flurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen of 0.95, 0.38 and 0.32 respectively Among the potential causes of this phenomenon are unequal rates of formation of the two derivatives (i.e. kinetic resolution) and unequal response by the detector employed. However, when UV absorption at 254nm was used to monitor the column eluate, it was found that the difference between the diastereomeric responses were negligible in all the cases, with the peak areas for each pair of analyte derivatives essentially reverting to a 1:1 ratio. It is clear therefore, that unequal peak areas were not the result of differing formation rates of the diastereomeric derivatives, but arise as a consequence of the diastereoisomers differing in their fluorescence properties. These observations were unexpected and the magnitude of the differences in fluorescence intensities were considerably greater than those of examples found in the literature. For instance, in the investigation of a wide range of drugs after derivatization with (-)-(*S*)-flunoxaprofen isocyanate reagent and using a fluorescence detector, the *R/S* peak-area ratios ranged between 0.97 and 1.03 in most instances, but the ratio was 1.23 for mexiletine and 1.09 for tranylcypromine (Martin *et al.*, 1989). In another study, the *R,R,R/S,R,R* peak-area ratio of the diacetyl tartrate derivatives of the enantiomers of propranolol was 1.08 when using a UV detector and 1.25 when a fluorescence detector was used (Linder *et al.*, 1989). Furthermore, diastereomeric isoindole derivatives formed by reaction of primary amine drugs with ortho-phthaldialdehyde and a homo-chiral thiol, often display unequal detector response and interesting, in several cases, unequal response was observed with UV detection rather than fluorescence detection (Desai and Gal, 1993).

Clearly an equal response to the derivatives is highly preferable, and this is one of the factors that make the direct approach to enantiospecific analysis superior in principle to the indirect method. It should be noted, however, that an unequal detector response does not necessarily render an indirect separation invalid because in quantitative analysis it is usually necessary to base the quantification on individual calibration curves

for the diastereomeric derivatives of the analyte enantiomers. Nevertheless in this case, the substantial, rather than subtle, differences observed in response between the diastereoisomeric pairs would make the approach seem impractical and analytical validation more complex, even if it were possible to separate all the derivatives in a single chromatographic run.

4.3.2 Chiral mobile phase additive method

The introduction of a chiral selector into the mobile phase of an achiral chromatographic system offers a number of benefits for enantiomeric analysis including flexibility, a wide choice of possible additives, avoidance of the disadvantages associated with the indirect approach and such an approach is often more cost-effective than an equivalent chiral stationary phase. Cyclodextrins are cyclic chiral oligomers of D-(+)-glucose bonded thorough α-(1,4) linkages and are extensively used as chiral mobile phase additives. The secondary hydroxyl groups are located on the wider side of the cavity and the primary hydroxyl groups on the narrower end. Thus, the cyclodextrin structure is shaped like a truncated cone with a relatively hydrophobic interior and a hydrophilic exterior as depicted in Figure 4.4. The most commonly used cyclodextrins are β-cyclodextrin (cycloheptamylose) and its derivatives.

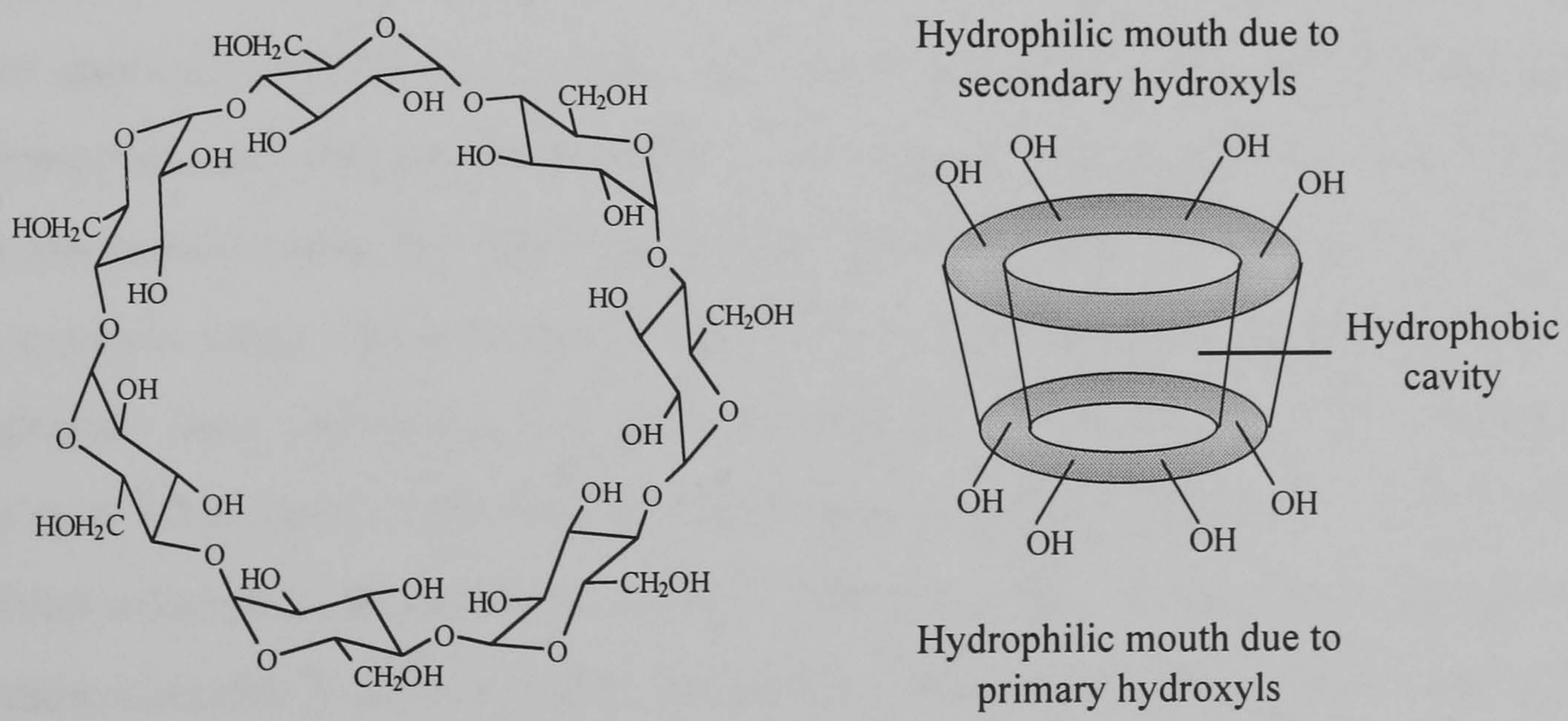


Figure 4.4: (a) Chemical structure and (b) toroidal shape of β-cyclodextrin.

However, the application of cyclodextrins as chiral additives for the resolution of 2-arylpropionic acid non-steroidal anti-inflammatory drugs has largely been in the field of capillary electrophoresis (Guttman and Cooke, 1994; Rawjee and Vigh, 1994; Fanali and Aturki, 1995; Fillet *et al.*, 1996; Nair and Armstrong, 1997; O'Keeffe *et al.*, 1997 and Zhu *et al.*, 1999), with limited use in liquid chromatography. The enantiomeric resolution of fenoprofen, ketoprofen and ibuprofen by HPLC was investigated, using native and derivatized β-cyclodextrins as chiral mobile phase additives by Ameyibor and Stewart (1997). Resolutions were achieved for fenoprofen and ketoprofen using a nonporous reversed-phase octadecylsilane column with hydroxypropyl-β-cyclodextrin (HP-β-CD) in the mobile phase, this approach was then used for the analysis of ketoprofen in human serum (Ameyibor and Stewart, 1998). The enantiomers of ibuprofen were only partially separated using this system and none of the compounds were resolved with native β-cyclodextrin (Ameyibor and Stewart, 1997). In reversed-phase applications, the resolution mechanism of β-cyclodextrins is thought to be the result of the formation of inclusion complexes in which the analyte is included into the cavity of the cyclodextrin. For enantiomers, resolution is possible if these inclusion complexes have different binding constants. In addition, the mouth of the cyclodextrin hydrophobic cavity is surrounded by secondary hydroxyl groups which are locked into position and are considered to be important in chiral recognition (Armstrong *et al.*, 1986 and Stalcup *et al.*, 1990). In the derivatized cyclodextrin some of these hydroxyls are substituted with freely rotating hydroxypropyl groups. This flexibility may allow for a closer approach between the hydroxyl groups and any hydrogen bonding moiety present in fenoprofen and ketoprofen, leading to stronger or more stereoselective interactions than are possible with the native β-cyclodextrin (Stalcup *et al.*, 1990). As well as the two aromatic rings which form inclusion complexes with the cyclodextrin, both these compounds have carboxylic acid groups at the chiral center that could participate in additional interactions with the rim hydroxypropyl groups. Ibuprofen is only partially resolved which may be due to its smaller molecular size, having a single aromatic ring structure, is unable to form a "tight" inclusion complex within the cyclodextrin cavity.

The structural similarities between flurbiprofen, and its metabolites, to fenoprofen and ketoprofen would suggest that HP-β-CD could also be applied for their enantiomeric resolution and so this was investigated using a short length Waters Symmetry C_{18} column. The column selected offers high efficiency and short analysis

times which is advantageous for minimising the organic modifier content of the mobile phase and thus circumventing problems associated with the limited solubility of cyclodextrins. The influence of organic modifier concentration, HP-β-CD concentration and different commercial preparations of HP-β-CD were investigated to try and achieve ideal separation characteristics.

The influence of the methanol content in the mobile phase on the retention and the enantioseparation of flurbiprofen and its metabolites is presented in Table 4.2. None of the analytes were baseline resolved with any of the mobile phase compositions investigated. However, partial resolution was observed for flurbiprofen and reduction of the alcohol content to 45% v/v or less resulted in limited enantioseparation of 3'-hydroxy-4'-methoxyflurbiprofen. A decrease in methanol concentration, as could be expected in reversed-phased systems, resulted in an increase in retention and also a slight improvement in enantioselectivity. It appears that at these high proportions, methanol acts as displacing agent from the hydrophobic environment of the cyclodextrin chiral cavity, thus weakening the strength of the inclusion complexes formed between the analytes and the cyclodextrin (Muñoz de la Peña *et al.*, 1991).

Table 4.2: Influence of the organic modifier concentrations on retention and enantioseparation of flurbiprofen and its metabolites on a Waters Symmetry C_{18} column *.

Methanol (% v/v)	Analyte	k'_1	k'_2	α	R_s
50	Flurbiprofen	15.28	15.41	1.01	< 0.2
	4'-Hydroxyflurbiprofen	3.13	3.13	1.00	-
	3'-Hydroxy-4'-methoxyflurbiprofen	4.70	4.70	1.00	-
45	Flurbiprofen	21.93	22.97	1.05	0.49
	4'-Hydroxyflurbiprofen	3.83	3.83	1.00	-
	3'-Hydroxy-4'-methoxyflurbiprofen	5.08	5.29	1.04	0.29
40	Flurbiprofen	25.97	29.61	1.14	0.64
	4'-Hydroxyflurbiprofen	4.61	4.61	1.00	-
	3'-Hydroxy-4'-methoxyflurbiprofen	6.15	6.45	1.05	0.37

** Mobile phase : aqueous TFA (pH 3.5; 0.1% v/v):methanol, containing HP-β-CD (1.1 % w/v)*

A chromatogram of the separation achieved using a mobile phase containing 40 % methanol outlining the lack of enantioseparation and also illustrating the relative long retention and poor peak shape, of the flurbiprofen enantiomers is shown in Figure 4.5. Investigations using lower methanol concentrations were not performed as this would have resulted in long run times with further band broadening of the flurbiprofen peaks. Substitution of the trifluoroacetic acid (0.1 % v/v) solution in the mobile phase with a phosphate buffer (pH 3.5, 0.01M) did not improve the separation or resolution profiles.

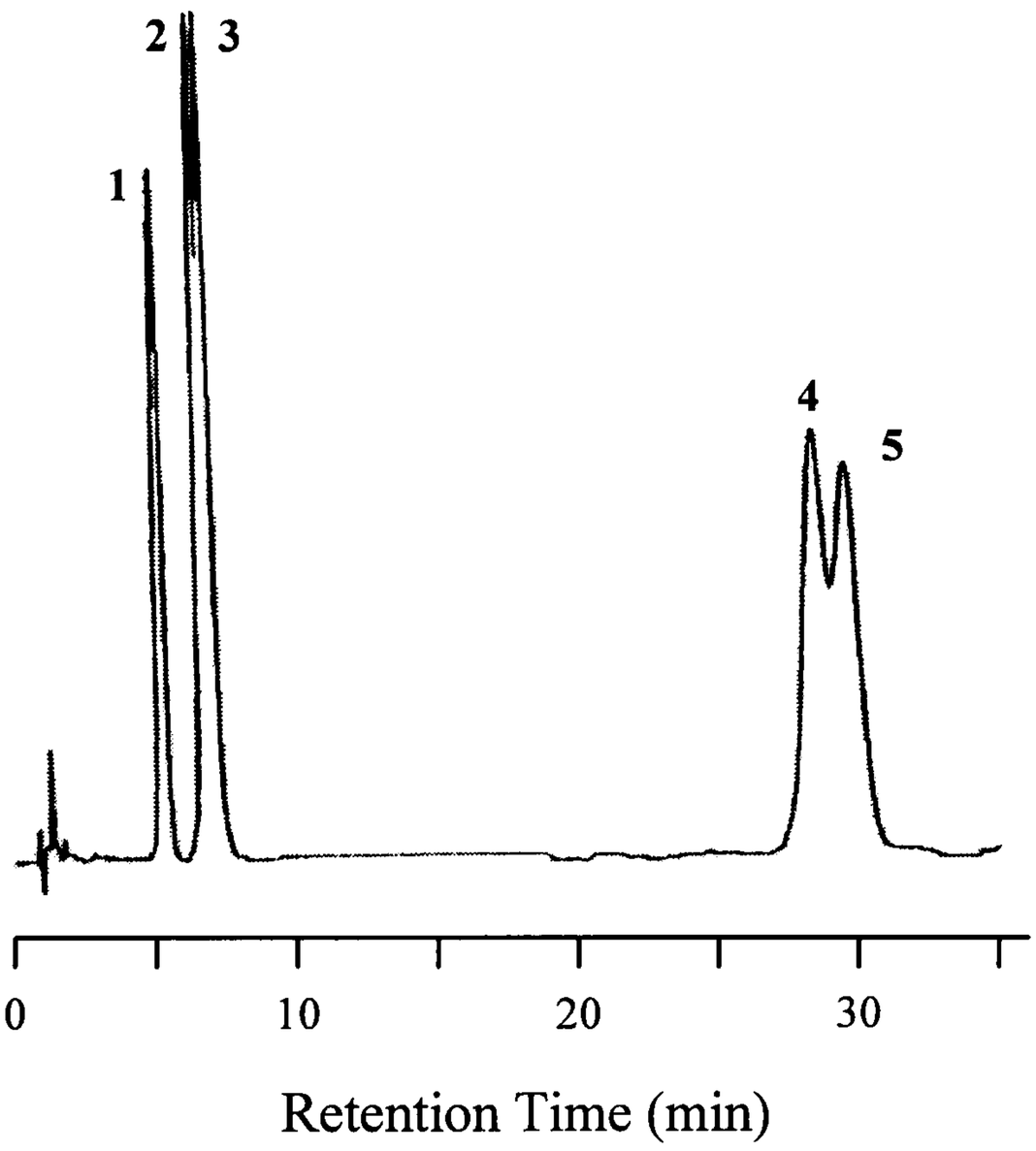


Figure 4.5: Chromatographic separation of flurbiprofen and its major metabolites using HP-β-CD as a mobile phase additive. Peaks : 1, (*R,S*)-4'-hydroxyflurbiprofen; 2 and 3, enantiomers of 3'-hydroxy-4'-methoxyflurbiprofen; 4, (*R*)-flurbiprofen; 5, (*S*)-flurbiprofen. [Stationary phase, Symmetry C_{18} column (75 x 4.6 mm, 3.5 μm); Mobile phase, aqueous TFA (pH 3.5; 0.1% v/v) : methanol (60:40 v/v) containing HP-β-CD (1.1 % w/v); Flow rate, 1.0 ml/min; Detection, UV λ = 254 nm].

The effect of HP-β-CD concentration in the mobile phase on the enantiomeric separation of the analytes is presented in Table 4.3. The best separation profile was observed using 1.1% v/v HP-β-CD in the mobile phase (Figure 4.5) with partial resolution of the enantiomers of flurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen. Further increases in the HP-β-CD concentration in the mobile phase resulted in faster elution of the analytes and a reduction in enantioseparation. Reduction in the mobile

phase content of HP-β-CD to less than 1.1% w/v increased the retention times of the analytes with little influence on resolution.

Table 4.3: Influence of the HP-β-CD concentrations on retention and enantioseparation of flurbiprofen and its metabolites on a Waters Symmetry C_{18} column *.

HP-β-CD (% w/v)	Analyte	k'_1	k'_2	α	R_s
0.9	Flurbiprofen	33.02	37.31	1.13	0.63
	4'-Hydroxyflurbiprofen	4.99	4.99	1.00	-
	3'-Hydroxy-4'-methoxyflurbiprofen	6.82	7.22	1.06	0.38
1.1	Flurbiprofen	25.97	29.61	1.14	0.64
	4'-Hydroxyflurbiprofen	4.61	4.61	1.00	-
	3'-Hydroxy-4'-methoxyflurbiprofen	6.15	6.45	1.05	0.37
1.3	Flurbiprofen	24.29	25.63	1.06	0.42
	4'-Hydroxyflurbiprofen	4.32	4.32	1.00	-
	3'-Hydroxy-4'-methoxyflurbiprofen	5.43	5.43	1.00	-
1.5	Flurbiprofen	19.51	20.51	1.05	0.36
	4'-Hydroxyflurbiprofen	4.12	4.12	1.00	-
	3'-Hydroxy-4'-methoxyflurbiprofen	5.08	5.08	1.00	-

** Mobile phase : aqueous TFA (pH 3.5; 0.1% v/v):methanol (60:40 w/v), containing HP-β-CD*

During the course of these studies, two other HP-β-CD preparations (denoted as preparations **II** and **III**) became available and their separation capabilities were compared to those of the original HP-β-CD (preparation **I**). As indicated in Table 4.4, there was considerable variability in the enantiomeric resolution capabilities of the different HP-β-CD preparations. Substituting HP-β-CD-**I** in the mobile phase with HP-β-CD-**II** resulted in an improved peak shape and resolution of the enantiomers of flurbiprofen (R_S = 0.84) but there was a complete loss of the partial resolution of 3'-hydroxy-4'-methoxyflurbiprofen. Using HP-β-CD-**III** in the mobile phase resulted in a loss of resolution for both flurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen. It is not surprising that these different preparations exhibited differing enantioresolution properties since HP-β-CDs are not pure compounds but impure mixtures of closely related homologs and isomers. The variability in the degree of substitution between

derivatized cyclodextrin preparations has been shown to have a significant effect on resolution of enantiomers (Valko *et al.*, 1994 and Szeman *et al.*, 1996). When using HP-β-CD as a CMPA it has been found that as the degree of substitution on the derivatized cyclodextrin increases, it can sometimes affect the binding process as well as the enantioselectivity. It is probable that the hydroxypropyl groups partially occlude the mouth of the cyclodextrin cavity and sterically influence the formation of inclusion complexes (Stalcup *et al.*, 1990). It is interesting to note that the descriptions supplied with HP-β-CD preparations **II** and **III**, provided no details with regards to the degree of substitution.

Table 4.4: Influence of different HP-β-CD preparations on retention and enantioseparation of flurbiprofen and its metabolites on a Waters Symmetry C_{18} column *.

HP-β-CD preparation †	Analyte	k'_1	k'_2	α	R_s
I	Flurbiprofen	25.97	29.61	1.14	0.64
	4'-Hydroxyflurbiprofen	4.61	4.61	1.00	-
	3'-Hydroxy-4'-methoxyflurbiprofen	6.15	6.45	1.05	0.37
II	Flurbiprofen	25.94	27.64	1.07	0.84
	4'-Hydroxyflurbiprofen	5.84	5.84	1.00	-
	3'-Hydroxy-4'-methoxyflurbiprofen	6.03	6.03	1.00	-
III	Flurbiprofen	19.32	19.32	1.00	-
	4'-Hydroxyflurbiprofen	5.16	5.16	1.00	-
	3'-Hydroxy-4'-methoxyflurbiprofen	5.64	5.64	1.00	-

**Mobile phase : aqueous TFA (pH 3.5; 0.1% v/v):methanol (60:40 w/v) with HP-β-CD (1.1 % w/v)*

† HP-β-CD preparations: I = Aldrich (Molar sub. = 0.6, average MW = 1380)

II = Wacker Chemicals Ltd. (Pharmaceutical grade)

III = Wacker Chemicals Ltd. (Standard grade)

In conclusion the use of HP-β-CD as a CMPA for the enantiomeric resolution of flurbiprofen and its metabolites proved to be of little success with none of the analytes being baseline resolved. However, these investigations did highlight the variability in enantioselectivities between different HP-β-CD preparations and hence the importance of using well characterised cyclodextrins in the development of separation methods. In addition, it is more than likely that better enantioseparation would have been observed if

a octyladecylsilane phase that required relatively low organic modifier concentrations had been available; for instance the novel nonporous column used by Ameyibor and Stewart (1997) was only able to demonstrate enantioselectivity for fenoprofen and ketoprofen when the acetonitrile concentration in the mobile phase was less than 6%. The use of native β-cyclodextrin as a CMPA, instead of HP-β-CD, showed no resolution capabilities for any of the three analytes (unpublished).

4.3.3 Chiral-AGP column

α_1-Acid glycoprotein (AGP), or orosomucoid, is a human plasma protein having a molecular weight of 41,000 to 45,000 and consists of a linear polypeptide with branching chains of carbohydrate. It has been demonstrated that immobilised AGP contains one high affinity binding site and at least one other site which binds the solute molecules with lower affinity (Enquist and Hermansson, 1990). The binding sites are built up by the peptide chain and they contain a large number of potential chiral binding moieties. The presence of different types of highly stereoselective interaction sites renders these CSPs very versatile, but the loading capacity is low due to the limited number of interaction sites (Hermansson and Schill, 1988). Several kinds of interaction can be involved in the retention mechanism, such as hydrophobic, hydrogen-bonding and ionic interactions (Schill *et al.*, 1986; Hermansson and Schill, 1988; Allenmark, 1989).

The use of an AGP-column for the resolution of 2-arylpropionic acids was initially investigated by Hermansson and Eriksson (1986). With the use of *N,N*-dimethyl-octylamine (DMOA) as a charged modifier in 2% v/v isopropanol (IPA) in phosphate buffer, enantiomeric resolution of ibuprofen, ketoprofen and naproxen was achieved. AGP columns have been used in stereospecific assays for fenoprofen (Menzel-Soglowek *et al.*, 1990), flurbiprofen (Geisslinger *et al.*, 1992), ibuprofen (Menzel-Soglowek *et al.*, 1990; Petterson and Olsson 1991; de Vries *et al.*, 1994), ketoprofen (Menzel-Soglowek *et al.*, 1990), naproxen (Andersen and Hansen, 1992) and the taurine conjugate of 2-[4-(3-methyl-2-thienyl)phenyl]propionic acid, a new orally effective anti-inflammatory agent, in dog urine (Konishi *et al.*, 1998). The fact that this CSP is used in the reversed-phase mode offers the possibility of direct injection of aqueous samples but moreover allows for greater flexibility in the mobile phase composition to try and in order to obtain baseline resolution (Hermansson and Eriksson, 1986). The effects of pH, DMOA

concentration and the content of IPA were investigated to optimise enantiomeric resolution of flurbiprofen and its metabolites on a Chiral-AGP column.

The influence of the mobile phase pH on the enantioseparation of flurbiprofen and its metabolites is presented in Table 4.5. With the mobile phase conditions investigated, there was no resolution of 3'-hydroxy-4'-methoxyflurbiprofen and partial resolution of 4'-hydroxyflurbiprofen. Flurbiprofen eluted considerably later than its metabolites and demonstrated baseline resolution at all pH values investigated. However, (*S*)-flurbiprofen, the later eluting peak, always had a retention time greater than 70 minutes and tended to exhibit extremely poor peak shape with increased broadness and loss of symmetry. Thus monitoring chromatographic performances was restricted to an arbitrary period of 70 minutes.

Table 4.5: Influence of the mobile phase pH on retention and enantioseparation of flurbiprofen and its metabolites on a Chiral-AGP CSP *.

pH	Analyte	k'_1	k'_2	α	R_s
7.0	Flurbiprofen	13.74	N.D.	-	-
	4'-Hydroxyflurbiprofen	2.35	2.87	1.22	1.07
	3'-Hydroxy-4'-methoxyflurbiprofen	5.03	5.03	1.00	-
6.5	Flurbiprofen	27.10	N.D.	-	-
	4'-Hydroxyflurbiprofen	4.82	5.40	1.12	0.84
	3'-Hydroxy-4'-methoxyflurbiprofen	7.20	7.20	1.00	-
6.0	Flurbiprofen	38.45	N.D.	-	-
	4'-Hydroxyflurbiprofen	6.05	6.84	1.13	0.72
	3'-Hydroxy-4'-methoxyflurbiprofen	9.96	9.96	1.00	-

** Mobile phase : phosphate buffer (0.01M) :IPA (99:1 v/v) containing 1.0 mM DMOA.*

N.D. = not determined, since retention time > 70 minutes.

As can be seen from the data presented in Table 4.5, the capacity factors of the analytes increased with decreasing pH from 7.0 to 6.0. It is not surprising that pH is a fundamental parameter when using a Chiral AGP since under the pH range investigated both the analytes and the AGP will be charged. AGP is an acidic protein with an isoelectric point of 2.7 and so decreasing the pH generates a larger number of positive charges (protonated amino groups) within the protein and therefore carboxylate anions are retained more strongly (Hermansson and Schill, 1988; Allenmark, 1989). It has also

been postulated that a change of pH can give rise to conformational changes that can have a strong effect on the bonding properties of the protein (Hermansson and Schill, 1988). The change in pH did not seem to have a significant influence on the enantioselectivity of 4'-hydroxyflurbiprofen, which is consistent with the findings observed for other 2-arylpropionic acids (Hermansson and Eriksson, 1986).

In an attempt to improve the separation profile the concentration of DMOA, a cationic modifier, in the mobile phase at pH 6.5 was increased from 1.0 mM to 5.0 mM. The increase in DMOA concentration resulted in faster elution of the analytes with a modest improvement on the enantioselectivity of 4'-hydroxyflurbiprofen and a slight decrease in the resolution factor (Table 4.6).

Table 4.6: Influence of the DMOA concentration on retention and enantioseparation of flurbiprofen and its metabolites on a Chiral-AGP CSP *.

DMOA (mM)	Analyte	k'_1	k'_2	α	R_s
1.0	Flurbiprofen	27.10	N.D.	-	-
	4'-Hydroxyflurbiprofen	4.82	5.40	1.12	0.84
	3'-Hydroxy-4'-methoxyflurbiprofen	7.20	7.20	1.00	-
5.0	Flurbiprofen	19.83	N.D.	-	-
	4'-Hydroxyflurbiprofen	4.36	5.49	1.26	0.62
	3'-Hydroxy-4'-methoxyflurbiprofen	5.65	5.65	1.00	-

** Mobile phase : phosphate buffer (pH 6.5, 0.01M) :IPA (99:1 v/v) containing DMOA.*

N.D. = not determined, since retention time > 70 minutes.

These finding are consist with those previously observed by Hermansson and Eriksson (1986) and Hermansson and Hermansson (1994), who showed for a series of 2-aryl-propionic acids that the k'_1-values were unchanged or slightly reduced with increasing DMOA concentration, whereas all k'_2-values increased, yielding improved enantioselectivity. It is postulated that solutes are distributed as ion-pairs with DMOA to the stationary phase and that the bulky DMOA ion-pair of one enantiomer in a pair fits better in the chiral binding site of the protein due to conformational reasons (Hermansson and Eriksson, 1986). However, it should be noted that decrease in retention times with increasing concentrations of ion-pairing agents has also been observed using other protein-based columns (Ceccato *et al.*, 1998). Thus, the effect from

ion-pairing (charged) mobile phase additives on *k'*- and α-values is rather difficult to predict.

The presence of IPA, an uncharged modifier, in the mobile phase is essential to reduce the degree of hydrophobic interactions between the analytes and the AGP (Enquist and Hermansson, 1990). As reported in Table 4.7, increasing the content of IPA resulted in faster elution of the analytes and decreased enantioselectivity. Having 1.5% v/v IPA in the mobile phase reduced the retention time of (*S*)-flurbiprofen to less than 70 minutes. A further increase to 5% resulted in a dramatic decrease in capacity factors, with (*S*)-flurbiprofen having a retention time of only 13.6 minutes and still exhibiting baseline resolution from its antipode, but such conditions result in insufficient retention of the enantiomers of 4'-hydroxyflurbiprofen and resolution is lost for this analyte. To obtain acceptable enantiomeric resolutions for both flurbiprofen and 4'-hydroxyflurbiprofen in a single isocratic run would require the use of 0.3% v/v IPA in the mobile phase, however the last eluting peak has a retention time of *ca.*140 minutes and shows poor peak shape (Figure 4.6).

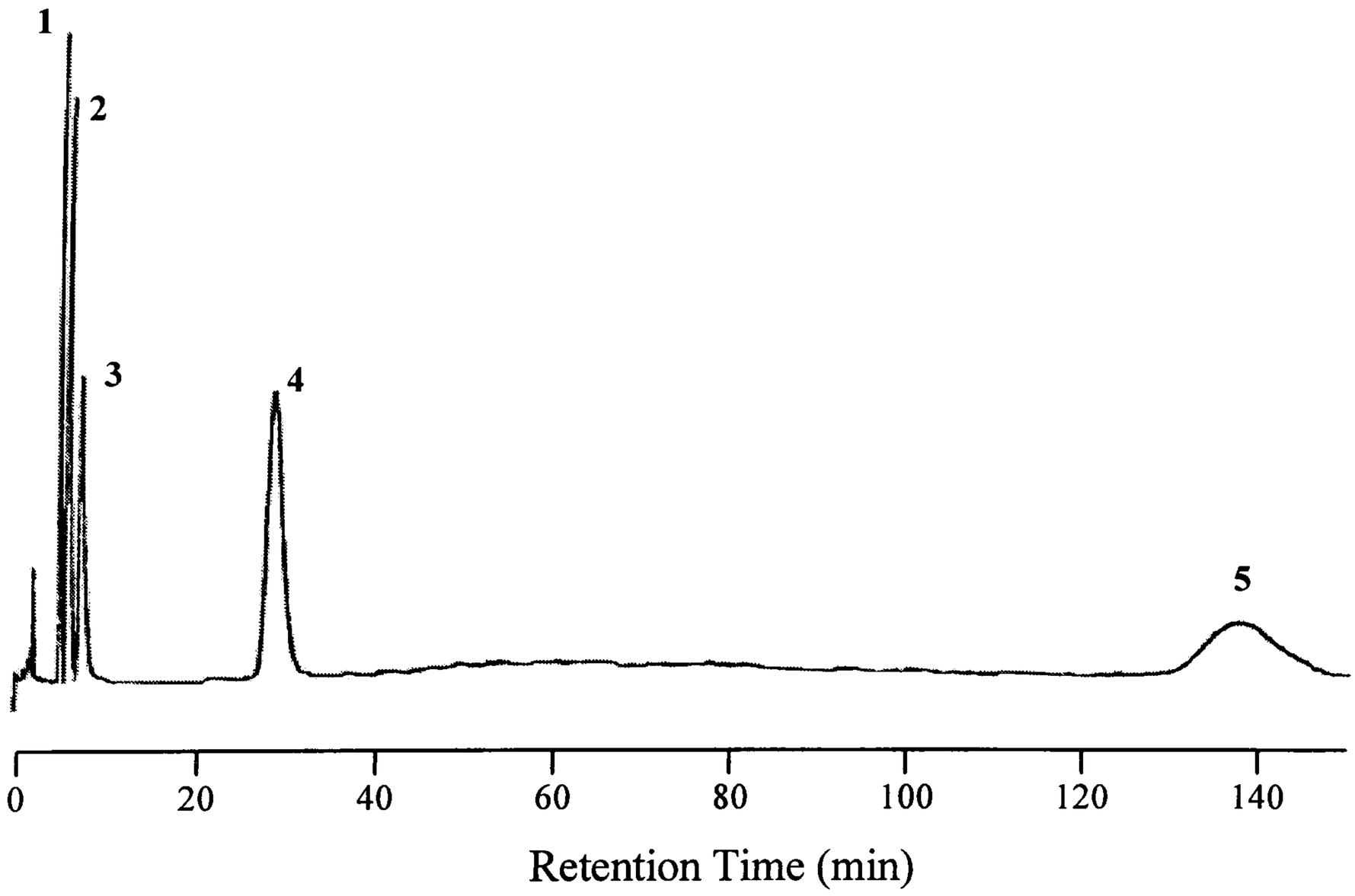


Figure 4.6: Chromatographic separation and resolution of flurbiprofen and its major metabolites using a Chiral-AGP CSP. Peaks : 1 and 2, enantiomers of 4'-hydroxyflurbiprofen; 3, (*R,S*)-3'-hydroxy-4'-methoxyflurbiprofen; 4, (*R*)-flurbiprofen; 5, (*S*)-flurbiprofen. [Mobile phase, phosphate buffer (pH 6.5, 0.01M):IPA (99.7:0.3 v/v) containing 1.0 mM DMOA; Flow rate, 0.5ml/min; Detection, UV λ = 254 nm].

Clearly such conditions are not suitable for routine and quantitative applications. The use of alternative organic modifiers, such as acetonitrile and ethanol, did not improve the resolution for any analyte.

Table 4.7: Influence of the organic modifier concentration on retention and enantioseparation of flurbiprofen and its metabolites on a Chiral-AGP CSP *.

IPA (%)	Analyte	k'_1	k'_2	α	R_s
0.3	Flurbiprofen	31.52	180.00	5.71	9.00
	4'-Hydroxyflurbiprofen	4.89	6.55	1.34	1.27
	3'-Hydroxy-4'-methoxyflurbiprofen	7.53	7.53	1.00	-
1.0	Flurbiprofen	27.1	N.D.	-	-
	4'-Hydroxyflurbiprofen	4.82	5.40	1.12	0.84
	3'-Hydroxy-4'-methoxyflurbiprofen	7.20	7.20	1.00	-
1.5	Flurbiprofen	19.84	77.97	3.93	7.10
	4'-Hydroxyflurbiprofen	3.98	4.89	1.23	0.65
	3'-Hydroxy-4'-methoxyflurbiprofen	6.52	6.52	1.00	-
3.0	Flurbiprofen	10.08	29.64	2.94	5.20
	4'-Hydroxyflurbiprofen	3.01	3.01	1.00	-
	3'-Hydroxy-4'-methoxyflurbiprofen	4.55	4.55	1.00	-
5.0	Flurbiprofen	6.59	16.79	2.56	2.51
	4'-Hydroxyflurbiprofen	2.05	2.05	1.00	-
	3'-Hydroxy-4'-methoxyflurbiprofen	3.60	3.60	1.00	-

** Mobile phase : phosphate buffer (pH 6.5, 0.01M) :IPA containing 1.0 mM DMOA*

N.D. = not determined, since retention time > 70 minutes.

In summary, it was not possible to obtain enantiomeric resolution of flurbiprofen and it's two major metabolites in a single chromatographic run using a Chiral-AGP CSP since this column was unable to discriminate between the enantiomers of 3'-hydroxy-4'-methoxyflurbiprofen. Furthermore the analysis time (150 minutes) required to obtain optimal enantioseparation of flurbiprofen and 4'-hydroxyflurbiprofen in a single run was not practical for enantiospecific bioanalysis. However, this problem could possibly be overcome by the use of gradient elution or by separately analysing flurbiprofen and 4'-hydroxyflurbiprofen. Typical chromatograms for the individual analysis of

flurbiprofen and 4'-hydroxyflurbiprofen with their optimised chromatographic conditions are presented in Figure 4.7. Of all the parameters investigated in an attempt to obtain the desired separation profile, only IPA concentration in the mobile phase had a significant influence on the enantioselectivity of flurbiprofen and 4'-hydroxy-flurbiprofen and thus it would seem that the hydrophobic interactions between the analyte and the AGP are associated with the chiral recognition process.

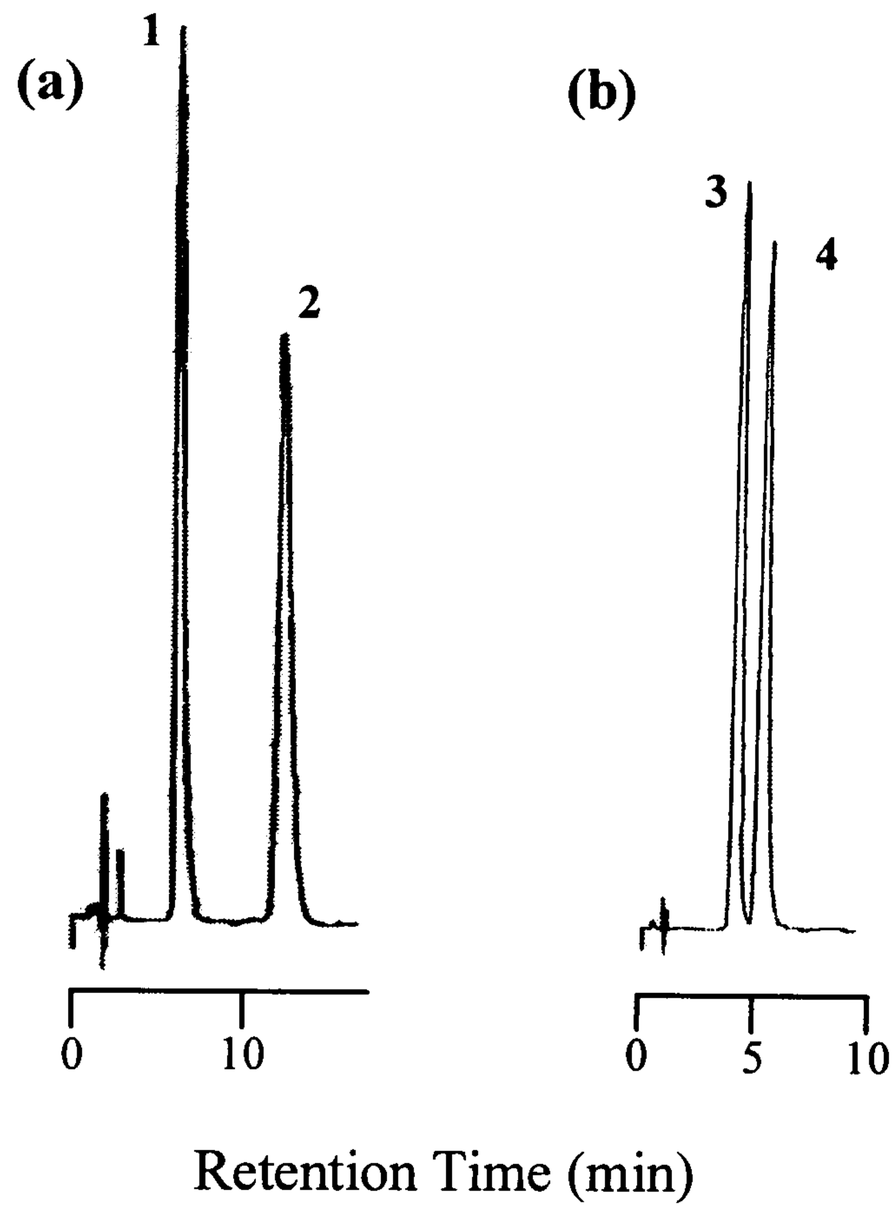


Figure 4.7: Chromatographic resolution using a Chiral-AGP CSP of (a) flurbiprofen. Peaks : 1, (*R*)-flurbiprofen; 2, (*S*)-flurbiprofen [Mobile phase, phosphate buffer (pH 6.5, 0.01M):IPA (95:5 v/v) containing 1.0 mM DMOA; Flow rate, 0.5ml/min; Detection, UV λ = 254 nm] and (b) 4'-Hydroxyflurbiprofen. Peaks : 3 and 4, enantiomers of 4'-hydroxyflurbiprofen [Mobile phase, phosphate buffer (pH 6.5, 0.01M):IPA (99.8:0.2 v/v) containing 1.0 mM DMOA; Flow rate, 0.5ml/min; Detection, UV λ = 254 nm].

4.3.4 Derivatized amylose (Chiralpak AD) CSP

Derivatized polysaccharide-based high-performance liquid chromatographic stationary phases are a key class of chiral stationary phases owing to their wide versatility and robustness (Okamato and Yashima, 1998). In particular, the CSP based on the tris (3,5-dimethylphenylcarbamate) derivative of amylose (Chiralpak AD) has been found to show high chiral recognition and been widely used to separate a broad range of racemic compounds and drugs (Yashima and Okamato, 1997; Okamoto and

Yashima, 1998). Derivatization of amylose with tris (3,5-dimethylphenylcarbamate) moieties facilitates the distribution of the solute to the CSP through hydrogen bonding to the amino and carbonyl groups and also via dipole-dipole interactions with the carbonyl group. In addition, the presence of the methyl substituents on the phenyl groups increase the electron density at the carbonyl oxygen atom of the carbamates and so encourage stronger hydrogen bonding interactions (Okamoto and Yashima, 1998). Besides these polar interactions, the π-π interaction between the phenyl group of the CSP and aromatic group of the analyte also play a significant role in chiral recognition (Okamoto and Yashima, 1998)

The use of an amylose tris (3,5-dimethylphenylcarbamate) (Chiralpak AD) phase for the chromatographic separation of 2-arylpropionic acid enantiomers was initially demonstrated by Okamoto *et al.* (1989), baseline separation was obtained for flurbiprofen, ketoprofen and tiaprofenic acid. However, the application of this CSP in bioanalysis has been confined to the analysis of ketoprofen in human plasma and urine (Carr *et al.*, 1995; Lovlin *et al.*,1996), analysis of tiaprofenic acid in plasma (Vakily and Jamali, 1996) and the stereospecific analysis of the major metabolites of ibuprofen in urine (Tan *et al.*, 1997b; see Chapter 2). The main disadvantage of this phase is the limited choice of mobile phases that can be used since chlorinated and aqueous solvents are prohibited as they may swell or dissolve the amylose-coat on the silica support (Enomoto *et al.*, 1996). Furthermore, biological sample work-up tends to be more critical to avoid the extraction of polar impurities that may interfere with the elution of the analytes or cause damage to the phase.

The optimal use of the Chiralpak AD phase requires an understanding of how chiral recognition occurs on this CSP. Booth and Wainer (1996) have described a "conformational driven" chiral recognition mechanism, rather than the standard "three-point interaction" model, for the separation of α-alkylarylcarboxylic acids on the Chiralpak-AD phase. The results from quantitative structure-enantioselective retention relationships and molecular modelling studies indicate that the enantioselective discrimination of the α-alkylarylcarboxylic acids on the AD-CSP is a three step process. These steps are : (a) "tethering" of the analyte to the CSP through hydrogen bonding interactions between the acid moiety of the analyte and the amine moieties on the CSP; (b) conformational adjustments of the analytes and insertion of the aromatic portion of the analyte into a "ravine" on the CSP; (c) stabilisation of the analyte-CSP complex via electrostatic and hydrogen bonding interactions within the "ravine". Both enantiomers

of the analyte form identical hydrogen bonding interactions and presumably the same hydrophobic interactions as well (Figure 4.8). Chiral discrimination arises due to the diastereomeric analyte-CSP complexes having different stabilities (Booth and Wainer, 1996).

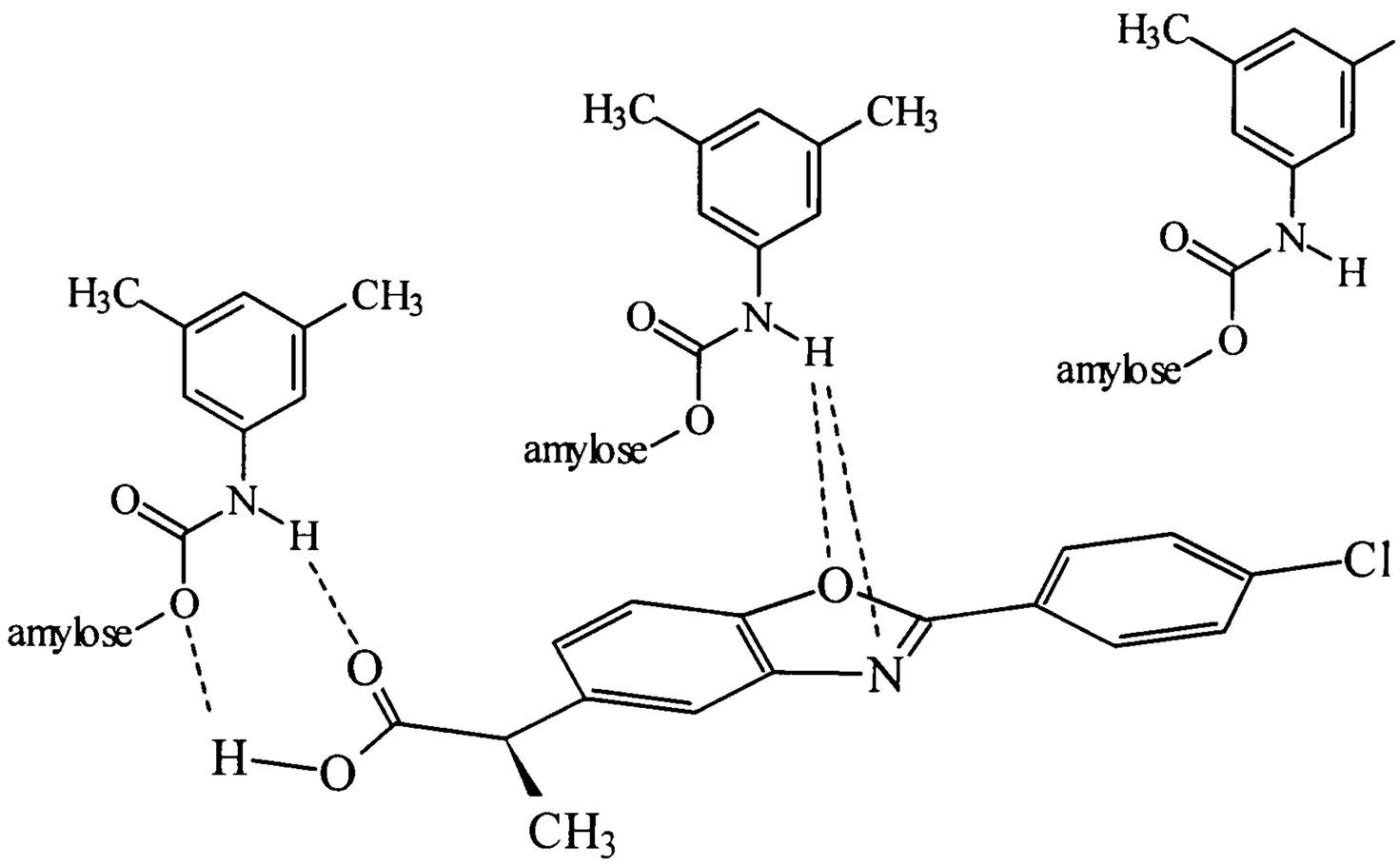


Figure 4 8: Proposed mechanism of binding between the amylose tris(3,5-dimethylphenyl-carbamate) chiral stationary phase and (*S*)-benoxaprofen, a model α-alkylarylcarboxylic acid (adapted from Booth and Wainer, 1996).

The separation of enantiomers on polysaccharide-based CSPs is essentially performed using a mobile phase of hexane containing an alcohol modifier, such as ethanol, methanol or isopropanol. The Chiralpak AD column that was to be evaluated for the analysis of flurbiprofen had previously been applied in our laboratory for the resolution of sulindac (Slováková *et al.*, 1998) and the separation of the stereoisomers of the metabolites of ibuprofen (Tan *et al.*, 1997b, 1997c; see Chapter 2). These separations had been performed using hexane-ethanol mixtures and required the addition of 0.05 % v/v trifluoroacetic acid (TFA) to suppress peak tailing and shorten the retention of the acidic drug analytes (Tang, 1996). The TFA present in the mobile phase reduced the hydrogen-bonding interactions of the acidic moiety of the analyte with the free silanol groups on the silica surface and also suppressed ionisation of the moiety. Therefore, using the same mobile-phase components and by altering the ethanol content, the Chiralpak AD phase was investigated to accomplish enantiomeric resolution of flurbiprofen and its metabolites within a reasonable run time. The chromatogram obtained using the mobile phase of hexane:ethanol (90:10 v/v) containing trifluoroacetic acid (0.05% v/v) at a flow rate of 1.0ml/min is shown in

Figure 4.9. The three analytes, were separated and enantiomerically resolved with high separation and resolution factors (Table 4.8). Furthermore there was no co-elution between any of the analytes, which is often a problem since CSPs tend to have good enantioselectivity but poor chemical selectivity (Hutt and Patel, 1998). It is also noteworthy that as this CSPs is used under normal-phase conditions unlike the other approaches investigated, the polar metabolites elute after, rather than before, the parent compound. The overall run time was limited to about 45 minutes which is acceptable for application in bioanalysis.

Table 4.8: Chromatographic parameters for the enantioseparation of flurbiprofen and its metabolites on a Chiralpak AD column *.

Analyte	k'_1	k'_2	α	R_S
Flurbiprofen	0.68	1.16	1.71	1.67
4'-Hydroxyflurbiprofen	2.69	4.44	1.65	3.67
3'-Hydroxy-4'-methoxyflurbiprofen	7.13	10.30	1.44	3.44

** Mobile phase : hexane : ethanol (90:10 v/v) containing trifluoroacetic acid (0.05% v/v)*

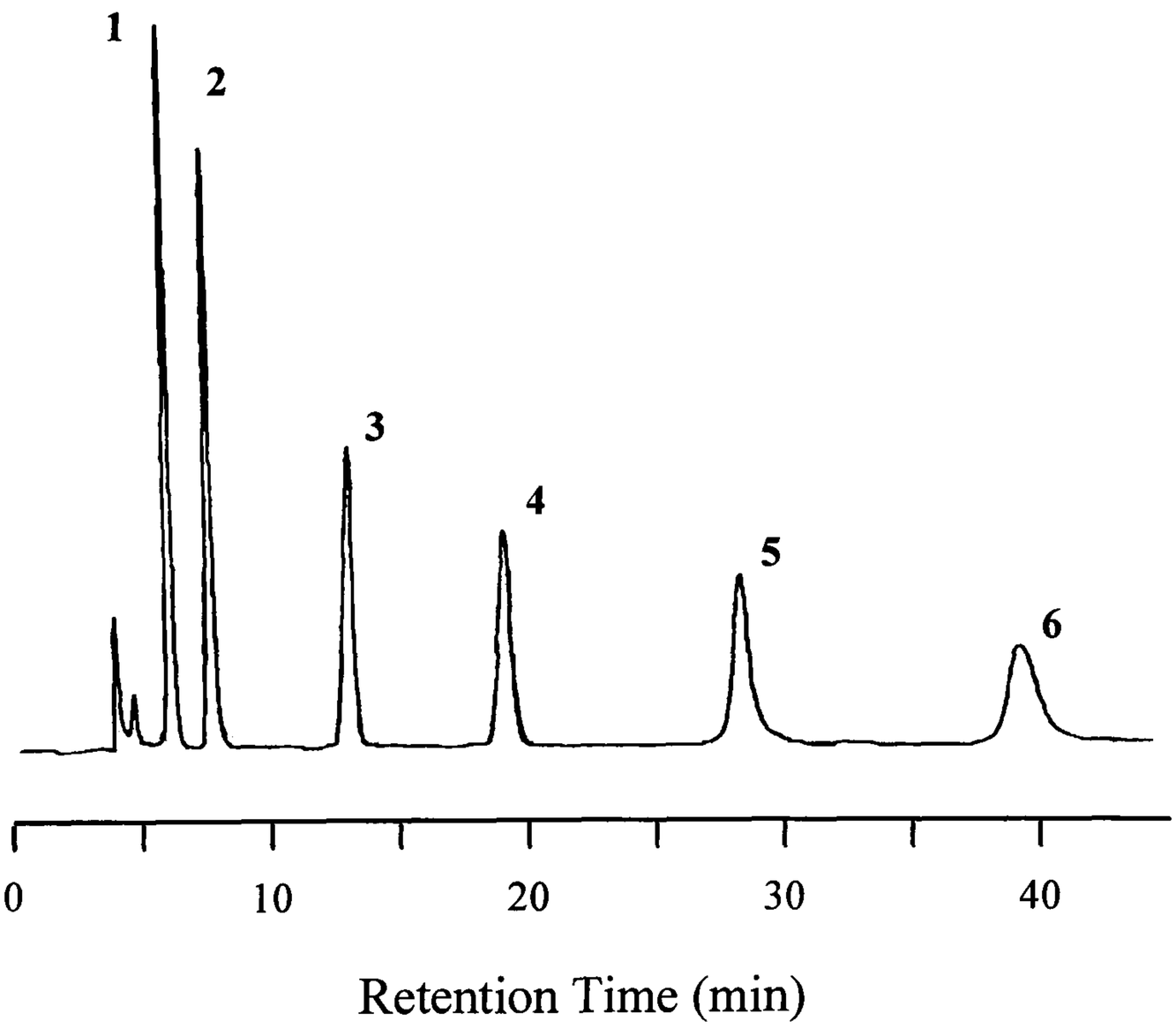


Figure 4.9: Chromatographic separation and resolution of flurbiprofen and its major metabolites using a Chiralpak AD CSP. Peaks : 1, (*R*)-flurbiprofen; 2, (*S*)-flurbiprofen; 3 and 4, enantiomers of 4'-hydroxyflurbiprofen; 5 and 6, enantiomers of 3'-hydroxy-4'-methoxyflurbiprofen. [Mobile phase hexane:ethanol (90:10 v/v) containing trifluoroacetic acid (0.05% v/v); Flow rate, 1.0ml/min; Detection, UV λ = 254 nm].

Thus it is clearly evident that the direct approach using the Chiralpak AD phase was the only one able to achieve the objective of baseline resolution of flurbiprofen and its metabolites in a single isocratic run and thus worthy of further evaluation for its application in bioanalytical studies (see Chapter 5). For such use it is essential that the stereochemical elution order of the analytes is established. However there is a lack of authentic chemical standards of the individual enantiomers of 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen and therefore these needed to be isolated by semi-preparative chromatography and characterised by circular dichroism spectroscopy.

4.3.5 Semi-preparative chromatographic resolution of the enantiomers of 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen.

The semi-preparative isolation of the enantiomers of 4'-hydroxyflurbiprofen was performed using the Chiralpak AD column with a modification in the mobile phase composition. The ethanol content was increased to 12% in order to minimise the run time without significantly compromising the resolution, as indicated by α and R_S values of 1.62 and 1.85 respectively (Figure 4.10a). Following multiple injections (n=50) of 4'-hydroxyflurbiprofen (0.4 mg per injection) approximately 9 mg of each enantiomer was isolated. Repeated analysis on the CSP indicated 98.2 % and 98.3 % enantiomeric purities for the first- and second-eluting enantiomers respectively (Figure 4.10 b and c).

The individual enantiomers of 3'-hydroxy-4'-methoxyflurbiprofen were collected using the Chiralpak AD phase in a similar manner. The mobile phase employed had an ethanol content of 15 %, which is the upper limit for this CSP, and resulted in baseline separation of 3'-hydroxy-4'-methoxyflurbiprofen with α and R_S values of 1.45 and 2.43 respectively (Figure 4.11a). Similar yields were obtained as for 4'-hydroxyflurbiprofen and the first and second eluting enantiomers had enantiomeric purities of 99.1 % and 99.0 % respectively (Figure 4.11 b and c).

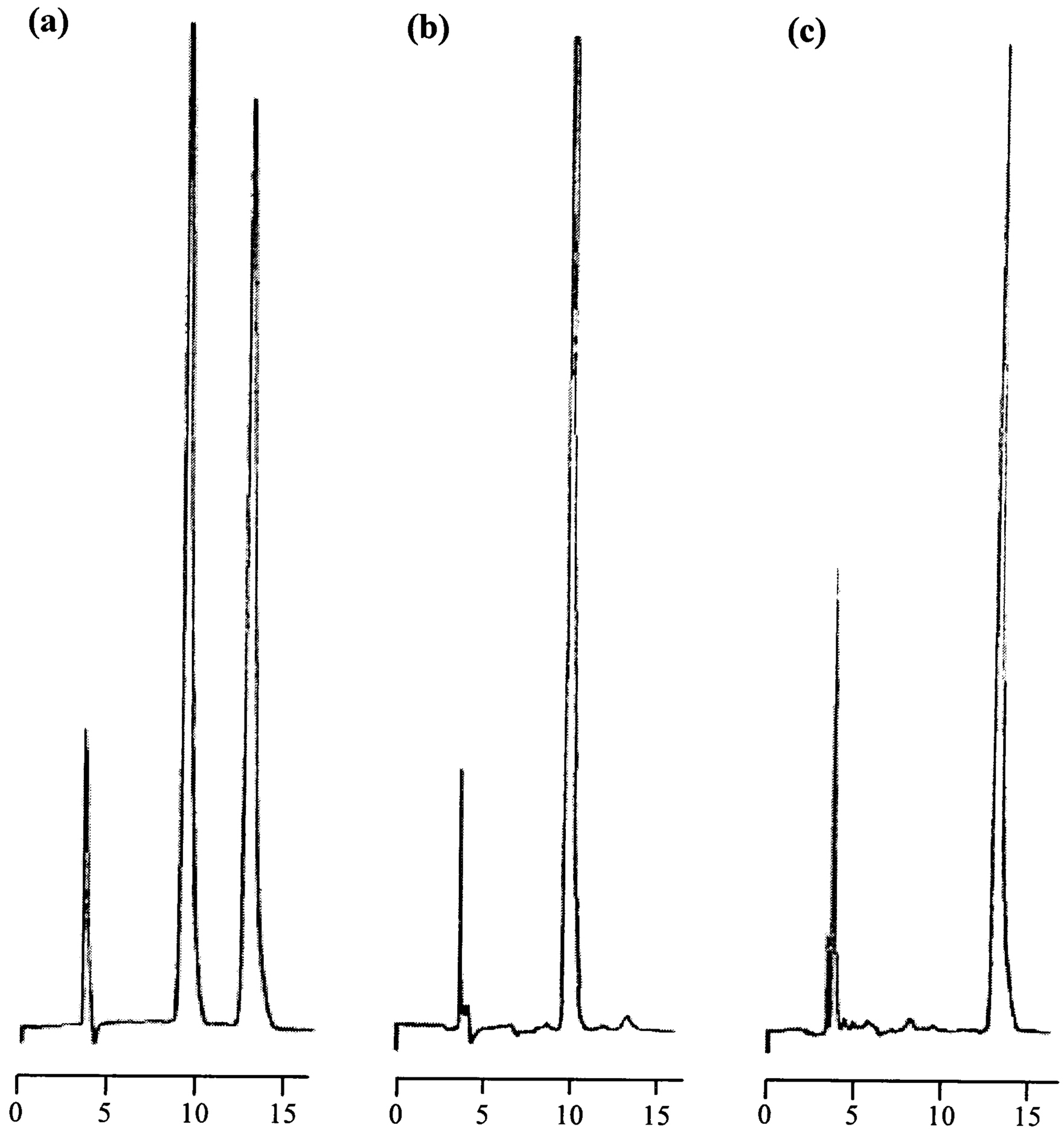


Figure 4.10: (a) Semi-preparative resolution of the enantiomers of 4'-hydroxyflurbiprofen using the Chiralpak AD CSP. Repeat chromatographic analysis of (b) first-eluting enantiomer and (c) second-eluting enantiomer [Mobile phase hexane:ethanol (87:13 v/v) containing trifluoroacetic acid (0.05% v/v); Flow rate, 1.0 ml/min; Detection, UV λ = 254 nm].

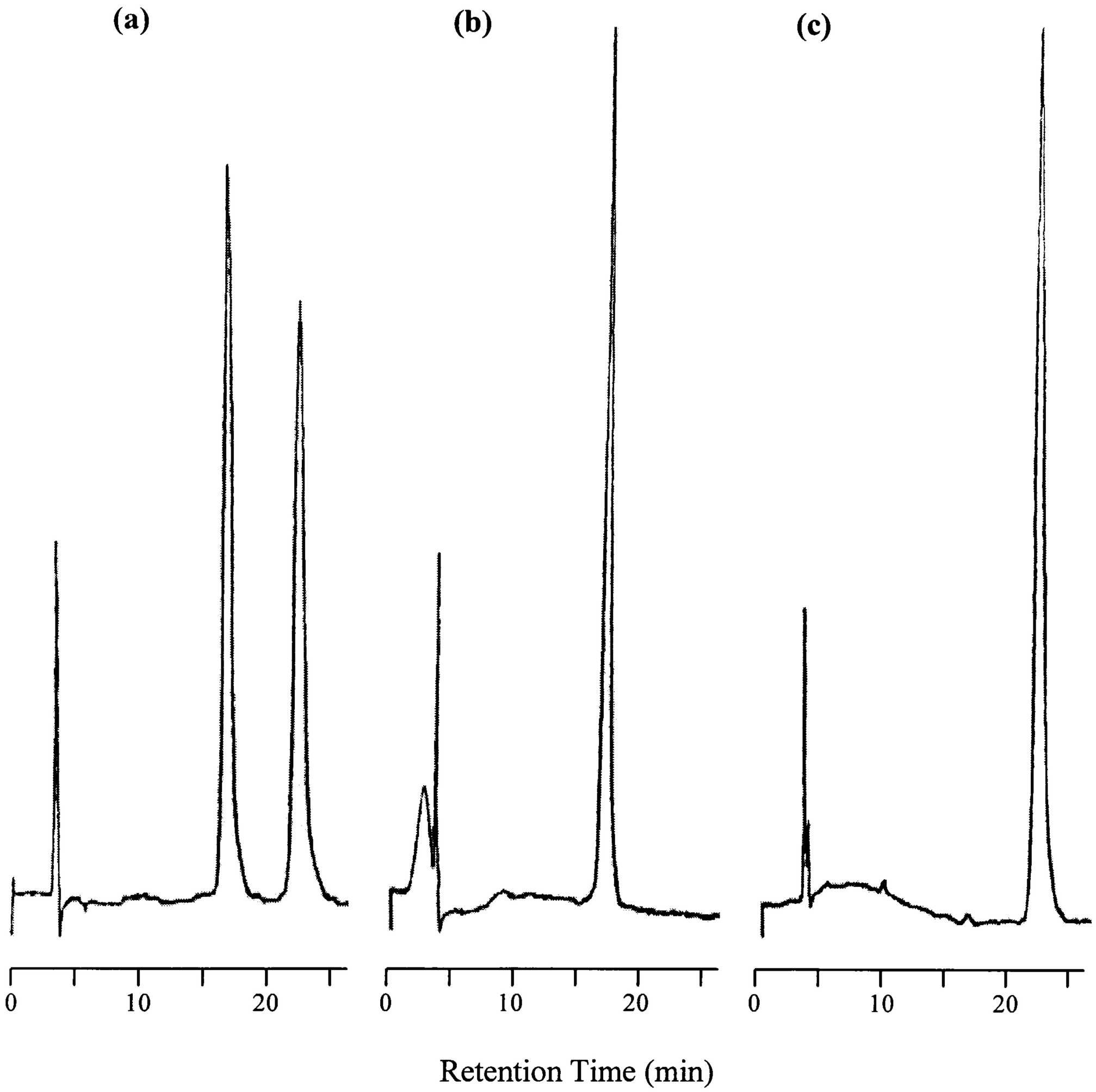


Figure 4.11: (a) Semi-preparative resolution of the enantiomers of 3'-hydroxy-4'-methoxy-flurbiprofen using the Chiralpak AD CSP. Repeat chromatographic analysis of (b) first-eluting enantiomer and (c) second-eluting enantiomer [Mobile phase hexane:ethanol (85:15 v/v) containing trifluoroacetic acid (0.05% v/v); Flow rate, 1.0 ml/min; Detection, UV λ = 254 nm].

4.3.6 Chiroptical properties of the enantiomers of 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen

An important aspect of assigning absolute configuration by circular dichroism (CD) is the spectroscopic assignment of the CD sign to a particular electronic transition. The UV absorption spectrum (Figure 4.12a) of (*R,S*)-flurbiprofen in acetonitrile is dominated by three $\pi \rightarrow \pi^*$ transitions at 275, 246 and < 210 nm which are associated with the biphenyl chromophore. The CD spectra (Figure 4.12b) provides evidence for optical activity at 275, 240, 220 and < 210 nm. The prominent 240 and 220 nm CD features must, by comparison with the UV absorption spectra, be associated with the carboxyl chromophore which is expected to have a minimal contribution to the UV spectrum. The sign of the CD of the carboxyl related transitions in α-alkylphenylacetic acid derivatives correlates with their absolute stereochemistry such that negative and positive signals correspond to the *R*- and *S*- absolute configurations respectively (Barth *et al.*, 1970). The negative and positive CD of (*R*)- and (*S*)-flurbiprofen (Figure 4.12b) correspond with those observed for (*R*)- and (*S*)-ibuprofen and related 2-arylpropionic acids (Barth *et al.*, 1970; Teulon *et al.*, 1978; Tan *et al.*, 1997c: Hoult *et al.*, 1999).

Hydroxylation of flurbiprofen to yield 4'-hydroxyflurbiprofen causes a red shift in the UV spectrum (Figure 4.12a) of the aromatic transitions with features at 285 and 258 nm and a shoulder appears at 218 nm. The wavelengths of the prominent CD features remain relatively unchanged (Figure 4.12c), the carboxyl assignment with CD sign in the 260 to 215 nm region remaining unaffected. The negative signal observed in this region for the first eluting enantiomer of 4'-hydroxyflurbiprofen and the positive signal seen with the second eluting enantiomer would suggest that the order of elution on the chiral stationary phase is (*R*)- before (*S*)-4'-hydroxyflurbiprofen, i.e. the same as that observed for flurbiprofen.

Unfortunately 3'-hydroxy-4'-methoxyflurbiprofen is not sufficiently soluble in acetonitrile and measurements had to be carried out in methanol. Optical activity can be very sensitive to solvent and stereochemical correlations need to be made with respect to the reference compound in the chosen solvent. The UV absorption spectrum of flurbiprofen was found to be effectively unchanged in either acetonitrile or methanol (compare Figures 4.12a and 4.13a). However, considerable changes are observed in the CD spectra (Figures 4.12b and 4.13b). For (*R*)-flurbiprofen the CD pattern remains positive at < 210 nm, negative at 220 nm and positive at 270 nm; however, the CD in

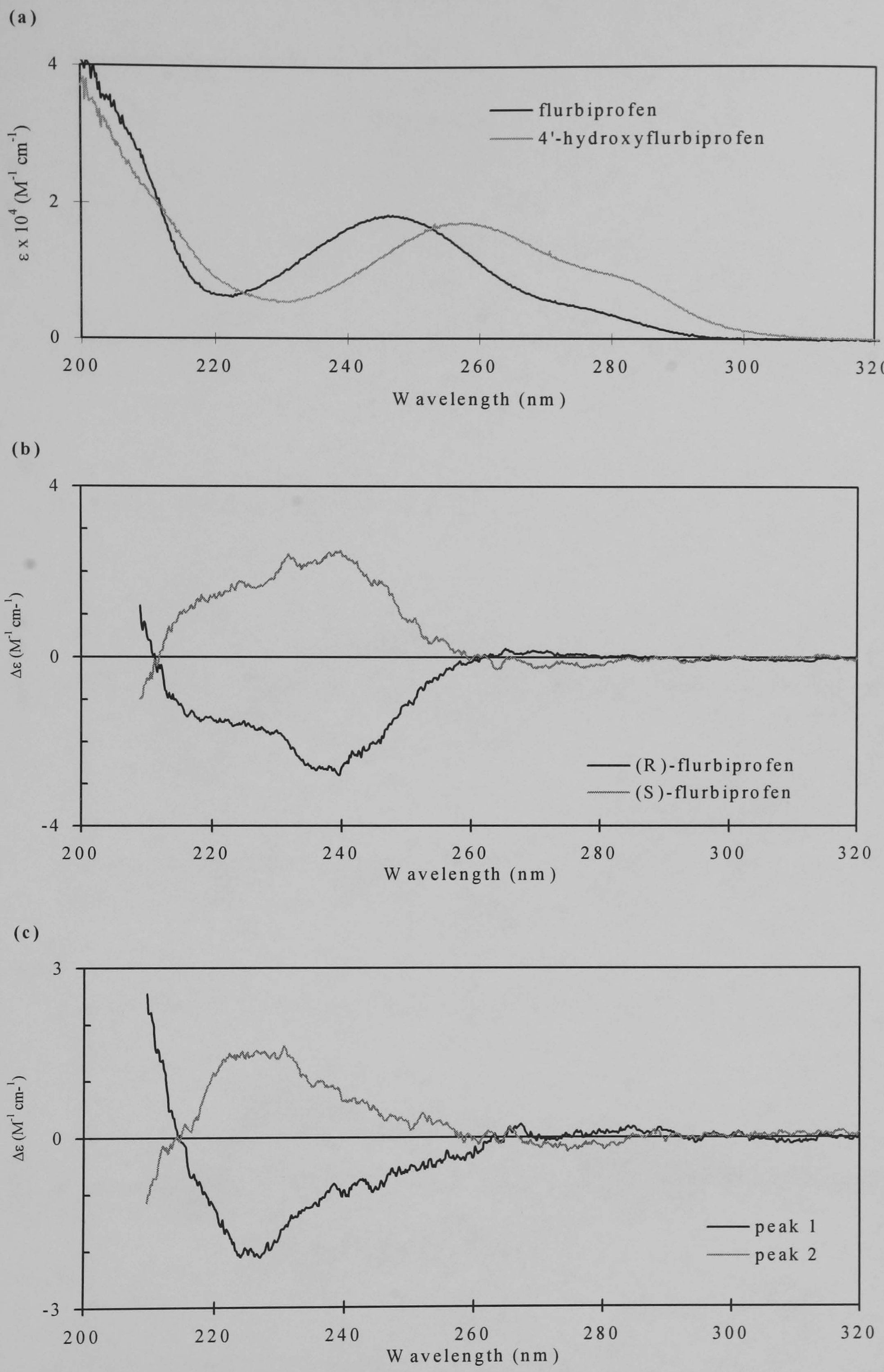


Figure 4.12: (a) UV spectra of flurbiprofen and 4′-hydroxyflurbiprofen; CD spectra determined in acetonitrile of (b) (*R*)- and (*S*)-flurbiprofen and (c) first and second eluting enantiomers of 4′-hydroxyflurbiprofen.

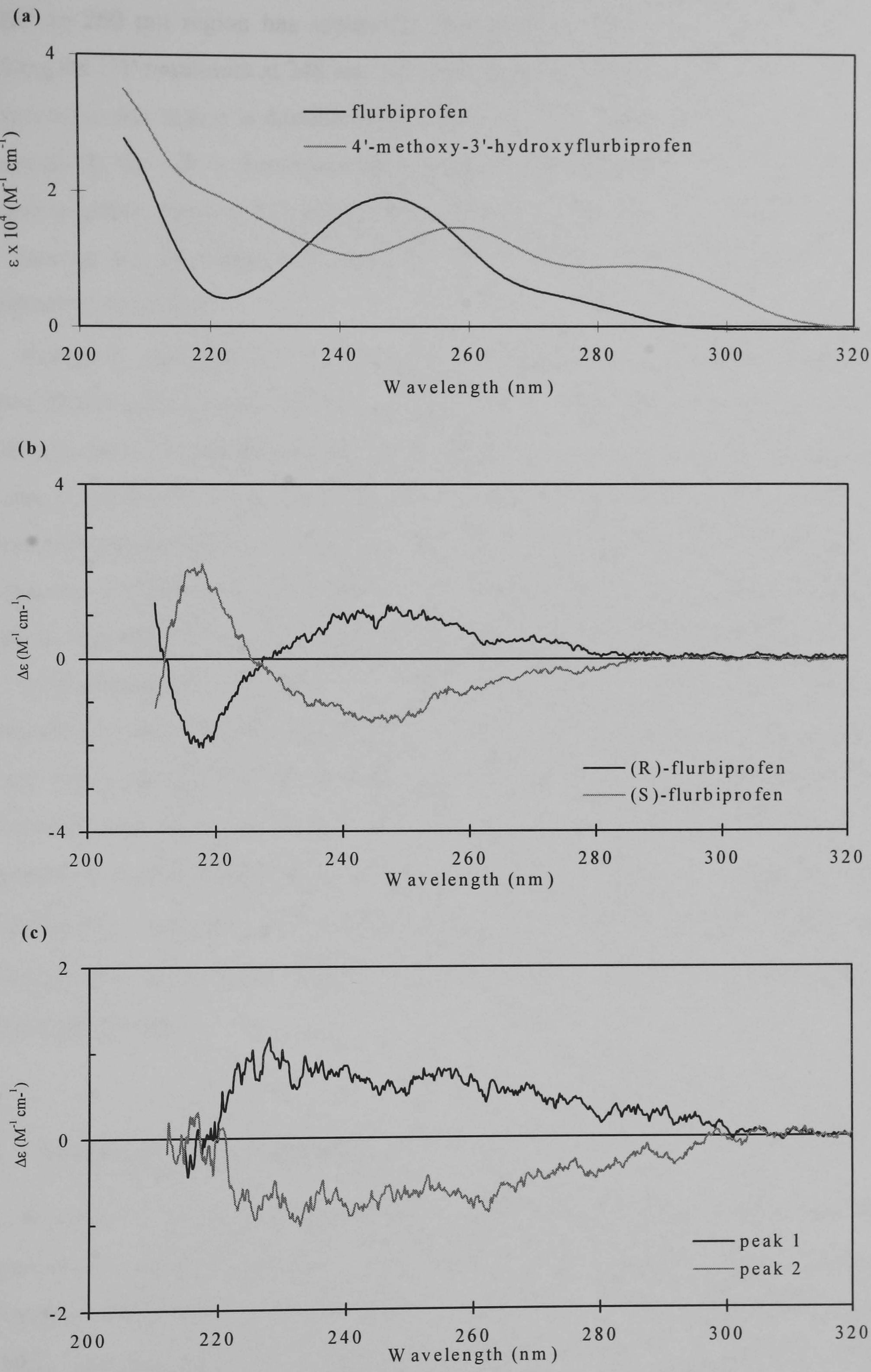


Figure 4.13: (a) UV spectra of flurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen; CD spectra determined in methanol of (b) (*R*)- and (*S*)-flurbiprofen and (c) first and second eluting enantiomers of 3'-hydroxy-4'-methoxyflurbiprofen.

the 225 to 260 nm region has apparently changed sign with a red shift to 248 nm matching the UV maximum at 248 nm. An obvious interpretation is that in methanol the CD spectra in this region is dominated by a positive biphenyl based transition whereas in acetonitrile the CD is dominated by a carboxyl based transition. This competition between negative carboxyl CD and positive biphenyl CD in the 225 to 260 nm region may account for the differences observed in acetonitrile between flurbiprofen and 4'-hydroxyflurbiprofen.

Increased substitution of the biphenyl ring system to form 3'-hydroxy-4'-methoxyflurbiprofen induces a further red shift of the UV absorption spectrum (Figure 4.13a) with features clearly observed at 292 and 260 nm and a more prominent signal at 225 nm. It appears that below 225 nm, the CD of 3'-hydroxy-4'-methoxyflurbiprofen is derived from two transitions: a carboxyl related signal remaining relatively unchanged at 219 nm and a biphenyl related signal of the opposite sign. As the biphenyl transition red-shifts with substitution, the CD in this region is effectively cancelled (Figure 4.13c). The configurational assignment of the enantiomers of 3'-hydroxy-4'-methoxy-flurbiprofen therefore has to be performed using a secondary comparison. The CD in the 260 nm region remains dominated by the biphenyl contribution as for the unsubstituted flurbiprofen and so by comparison of this region, the first eluting enantiomer of 3'-hydroxy-4'-methoxyflurbiprofen exhibits a positive signal as observed with (*R*)-flurbiprofen and the second-eluting enantiomer shows a negative signal like (*S*)-flurbiprofen. Thus the chromatographic elution order is (*R*)- before (*S*)-3'-hydroxy-4'-methoxyflurbiprofen.

4.4. Summary and conclusions

In order to separate the enantiomers of flurbiprofen, 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen using liquid chromatography, four different approaches were selected : reversed-phase HPLC after pre-column derivatization with (*R*)-NEA, reversed-phase HPLC using HP-β-CD as a CMPA, reversed-phase HPLC using an α_1-acid glycoprotein CSP (Chiral-AGP) and normal-phase HPLC using an amylose tris (3,5-dimethylphenylcarbamate) CSP (Chiralpak AD).

The (*R*)- 1-(naphthen-1-yl)ethylamide diastereoisomers of flurbiprofen and its two major metabolites could all be baseline resolved using a reversed-phase system.

However, this approach had the disadvantages that a run time greater than 45 minutes would have been required to achieve simultaneous separation and resolution of all three analyte derivatives and the use of fluorescence detection to enhance analytical sensitivity would not have been permitted due to the unequal florescent properties shown by diastereomeric derivatives.

The adoption of a direct method using HP-β-CD as a CMPA proved to be of limited success with only partial resolution observed for flurbiprofen and its 3'-hydroxy-4'-methoxy metabolite. This set of experiments also highlighted the lack of robustness of the approach with considerable variability observed in enantioselectivity between difference grades and brands of HP-β-CDs.

Baseline resolution of flurbiprofen and 4'-hydroxyflurbiprofen could be achieved using a Chiral-AGP CSP; but simultaneous analysis would have required a run time greater than 150 minutes, which is clearly not practical for quantitative and routine use. Furthermore, this column was unable to discriminate between the enantiomers of the 3'-hydroxy-4'-methoxy metabolite.

Of all the approaches, only the direct method using the Chiralpak AD CSP demonstrated separation and enantiomeric resolution of all three analytes within an acceptable run time of 45 minutes. Resolution values of 1.67, 3.67 and 3.44 were obtained for flurbiprofen, 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen respectively. Semi-preparative isolation of the individual enantiomers of both metabolites, followed by CD analysis, revealed that the elution order on the CSP was *R*- before *S*-enantiomer for both compounds and maintained the same order as that observed for flurbiprofen. The metabolite elution order was subsequently confirmed on the analysis of urine samples obtained from a healthy volunteer following oral administration of the individual drug enantiomers (see Chapter 5).

The above chiral-phase chromatographic system is the first to separate and resolve the enantiomers of flurbiprofen and its major metabolites in a single chromatographic run. The application of this methodology to the development of analytical methodology for the enantiospecific determination of flurbiprofen and its metabolites in urine and serum is described in the following Chapter.

CHAPTER 5 :

Stereospecific analysis of flurbiprofen and its two major metabolites in biological fluids

5.1. Introduction

Flurbiprofen, unlike ibuprofen, does not appear to undergo enantiomeric inversion in humans and is eliminated predominantly through metabolic oxidation and conjugation reactions (Risdall *et al.*, 1978; Szpunar *et al.*, 1987; Jamali *et al.*, 1988). Flurbiprofen undergoes oxidation to yield 4'-hydroxy- and 3',4'-dihydroxyflurbiprofen, the latter undergoing methylation to form 3'-hydroxy-4'-methoxyflurbiprofen, all of which retain the chiral centre (Figure 5.1). These metabolites, in addition to the parent compound are subjected to conjugation by either glucuronidation or in the case of the phenols, sulphation (Davies, 1995).

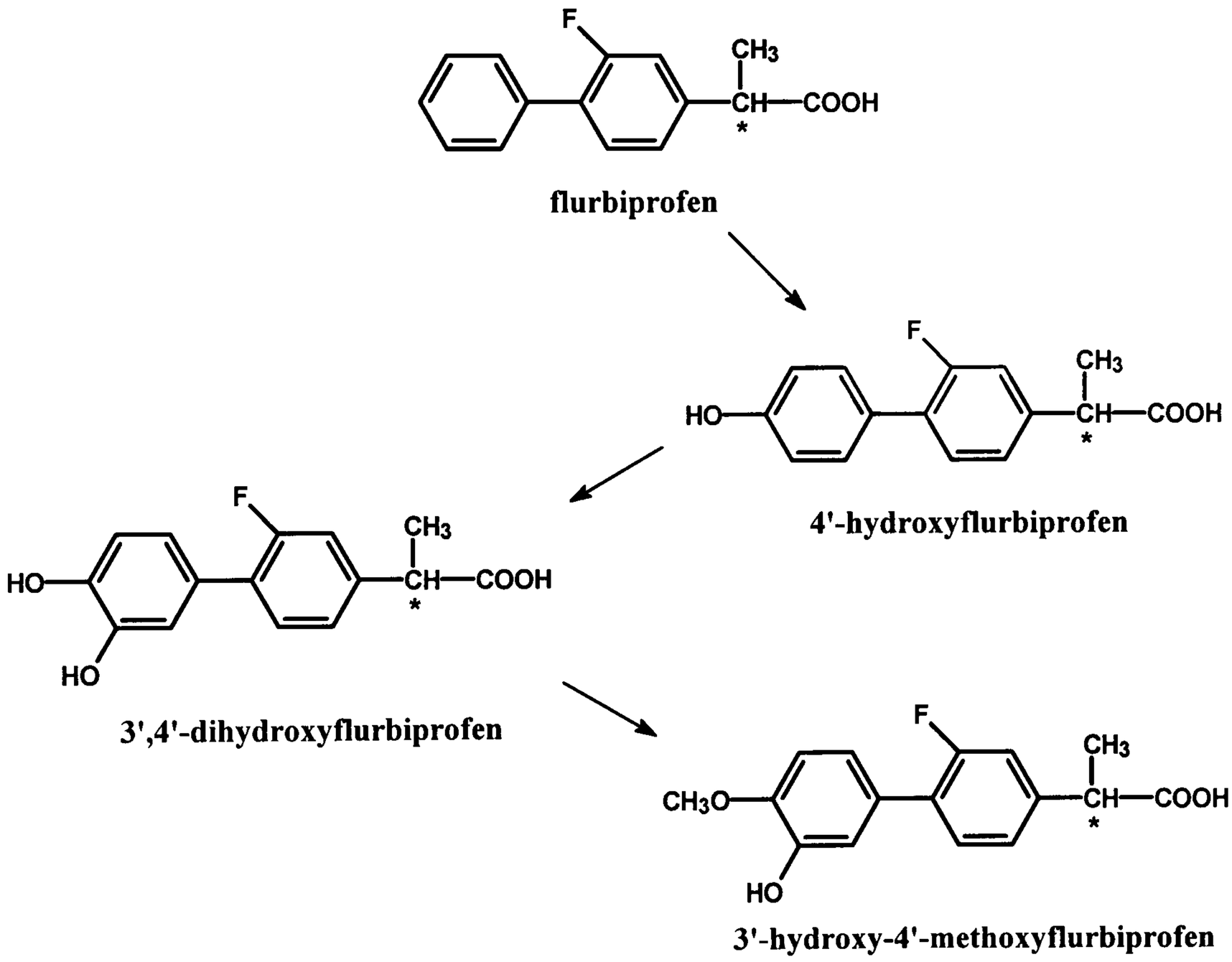


Figure 5.1: Metabolism of flurbiprofen in humans (* denotes chiral centre).

Urinary recovery studies by Szpunar *et al.* (1987) indicate that approximately 22% flurbiprofen, 48 % 4'-hydroxyflurbiprofen, 7 % 3'-hydroxy-4'-methoxyflurbiprofen and less than 1% 3',4'-dihydroxyflurbiprofen are excreted in urine, primarily as conjugates, following a 100mg oral dose. Comparable data was also obtained by Knadler and Hall (1989). Only flurbiprofen and 4'-hydroxyflurbiprofen have been detected in human serum or plasma, with the 4'-hydroxy metabolite being present at

concentrations 10 to 20 times less than those of the parent drug (Adams *et al.*, 1987; Szpunar *et al.*, 1987).

Clearly the pathways associated with the formation and elimination of these metabolites may exhibit enantioselectivity and therefore to obtain a comprehensive picture of the enantiospecific disposition of flurbiprofen, investigations should not be solely based on the quantitative analysis of the enantiomers of the parent drug but also of its metabolites. Having said that, the achiral chromatographic analysis of the metabolites of flurbiprofen in biological fluids has received little attention in the literature (Adams *et al.*, 1987; Szpunar *et al.*, 1987) and the development of enantiospecific methods been restricted due to the difficulties associated with the chromatographic separation and resolution of flurbiprofen and its metabolites, coupled with the lack of authentic standards of the individual enantiomers of the metabolites. The indirect approach based on HPLC adopted by Knadler and Hall (1989) for the analysis of the enantiomers of flurbiprofen and its metabolites in plasma and urine, circumvented chromatographic separation difficulties by employing different methods for the analysis of (*R*)- and (*S*)- flurbiprofen and for the enantiospecific analysis of 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen. Furthermore, the determination of flurbiprofen in plasma or urine required different conditions, the analysis of urine extracts required an adjustment in the mobile phase composition to allow for the simultaneous measurement of 3',4'-dihydroxyflurbiprofen and needed the use of fluorescence detection, rather than UV, to minimise interference from endogenous compounds. However, it is worth noting that UV detection was still essential for the monitoring of the internal standard in this assay.

The previous chapter outlined the chromatographic separation and resolution of flurbiprofen and its major metabolites, 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen on a amylose tris (3,5-dimethylphenylcarbamate) CSP (Chiralpak AD) and by a combination of semi-preparative isolation and chiroptical characterisation established the enantiomeric elution order for the metabolites. The ability to resolve all three analytes within a reasonable run time would indicate that this method is ideally suited for application in enantiospecific bioanalysis. This chapter therefore describes the development and validation of assays based on the Chiralpak AD CSP for the quantification of flurbiprofen and its metabolites in urine and serum. The utility of these assay methods for dispositional investigations was assessed by performing some preliminary urinary recovery studies following the oral administration of the drug to

three healthy male volunteers and the establishment of a serum concentration-time profile for one of these volunteers.

5.2. Experimental

5.2.1 Chemicals and reagents

Acetonitrile, dichloromethane, ethanol, ethyl acetate, hexane and isopropanol (HPLC grade) were purchased from Rathburn (Walkerburn, UK). Trifluoroacetic acid (TFA) and sodium hydrogen phosphate (GPR grade) were purchased from BDH (Poole, Dorset, UK). Sodium acetate pHix Buffer (4.0 M, pH 5.5 ± 0.03 at 25°C) was purchased from Pierce (Rockford, Illinois, USA). β-Glucuronidase (EC 3.2.1.31) type H-5, with 530 units of β-glucuronidase and 30 units of sulphatase activity per mg solid, was purchased from Sigma Chemicals (Poole, Dorset, UK). (*S*)-Naproxen was obtained from Aldrich Chemicals (Gillingham, Dorset, UK). (*R,S*)-Benoxaprofen was kindly supplied by Lilly Research Centre Ltd. (Windlesham, Surrey, UK) and (*R,S*)-, (*S*)-, (*R*)-flurbiprofen, (*R,S*)-4'-hydroxyflurbiprofen and (*R,S*)-3'-hydroxy-4'-methoxy-flurbiprofen were the generous gifts of Boots Company PLC (Nottingham, UK).

5.2.2 Chromatographic column

The Chiral stationary phase was a Chiralpak AD (amylose tris (3,5-dimethyl-phenylcarbamate)) column (250 x 4.6 mm, 10 μm) used with a matching guard column (50 x 4.6 mm, 10 μm) and was supplied by HPLC Technology Ltd. (Macclesfield, UK).

5.2.3 Instrumentation

Chiral-phase HPLC was performed using an LDC Constametric 3000 pump linked to a Kontron SFM-25 fluorescence detector (Watford, Herts., UK) and a LDC Spectromonitor 3100 UV detector integrator (Stone, Staffs., UK). Data from the two detectors was acquired using a LDC CI-4000 computing integrator and a LDC CI-4100 computing integrator (Stone, Staffs., UK). Samples were injected on column using a Perkin Elmer ISS-100 autosampler (Beaconsfield, Bucks., UK).

5.2.4 Enantiospecific analysis of flurbiprofen and its metabolites in urine

Sample preparation

Free: For the quantification of unconjugated flurbiprofen, 4'-hydroxy-flurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen, 1.0 ml aliquots of urine were used. To these samples were added 2.5 μg (50 μl of a 50 μg/ml solution in acetonitrile) of (*S*)-naproxen as internal standard. The samples were then acidified by the addition of hydrochloric acid (1.0 M; 100 μl) and buffered to pH 3.8 with 1.0 ml of phosphate buffer (pH 3.8; 1.0 M). Hexane:isopropanol (95:5, v/v; 6 ml) was added and the extraction tubes were then tightly capped and mixed on a test-tube rocker for 20 minutes. Phase separation was achieved by centrifugation for 5 minutes at 1000g. The organic layer was then separated into a clean glass tube and evaporated under a gentle stream of nitrogen at 40°C on a dry heating block. The residue was then reconstituted in 150 μl mobile phase and 100 μl injected into the chiral phase HPLC system.

Base hydrolysis: For the determination of the free plus acyl glucuronide concentrations of flurbiprofen, 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxy-flurbiprofen, 0.1 ml urine samples were used and diluted to 1.0 ml with distilled water. These samples were treated with sodium hydroxide (1.0 M; 200 μl) and the hydrolysis reaction was left to proceed for 2 hours at room temperature. Subsequently, the base was neutralised and the samples acidified by the addition of hydrochloric acid (1.0 M; 300 μl). Then (*S*)-naproxen (2.5 μg), 1.0 ml of phosphate buffer (pH 3.8; 1.0 M) and hexane:isopropanol (95:5 v/v; 6 ml) added before the samples were extracted as described above for the free samples. The quantity of an analyte excreted as acyl glucuronide was subsequently calculated by subtraction of the urinary concentration of the free analyte from the determined concentration following base hydrolysis.

Enzymatic and base hydrolysis: For the determination of total concentrations (i.e., free, acyl glucuronide and phenolic conjugates) of flurbiprofen, 4'-hydroxy-flurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen, 0.1 ml aliquots of urine were used. To these, for optimal hydrolysis of phenolic conjugates, were added β-glucuronidase (EC 3.2.1.31) type H-5 (1000 units of β-glucuronidase plus 57 units of sulphatase) in 0.9 ml acetate buffer (pH 5.0; 0.05 M) and incubated at 37°C for 16 hours. Following

this the samples were treated with NaOH (1.0 M; 200 µl) and the procedure followed as described above for base hydrolysis.

Optimisation of enzymatic hydrolysis conditions

Before determination of the conjugated levels of flurbiprofen and its metabolites in urine, optimal assay conditions for β-glucuronidase (EC 3.2.1.31) enzyme activity were established. This partially purified enzyme has 530 units of β-glucuronidase and 30 units of sulphatase activity per mg solid and, hereafter the activity of the enzyme system will be referred to solely in terms of β-glucuronidase activity to maintain simplicity.

In order to determine the appropriate amount of enzyme required, 0.1 ml samples, from a 4-6 hr urine collection obtained from a volunteer following the oral administration of 100 mg of the racemic drug, were incubated with 500, 1000, 2000, 4000 and 6000 units of β-glucuronidase in 0.9 ml acetate buffer (pH 5.0; 0.05 M). The hydrolysis was performed in duplicate at 37°C for a period of 4 hours and 24 hours for each of the enzyme concentrations. Enzyme activity was terminated by placing the incubates on ice and the samples prepared, as described above for free drug and metabolites, prior to analysis.

Upon establishing the most suitable quantity of enzyme (1000 units), a time course experiment was performed to determine the incubation time period which would be sufficient to completely liberate the phenolic and 1-*O*-acyl-glucuronides and sulphate conjugates of the drug and metabolites. Samples (0.1 ml) of the volunteer's 4-6hr urine collection were incubated in duplicate with 1000 units of β-glucuronidase in 0.9 ml acetate buffer (pH 5.0; 0.05 M) at 37°C for 0, 2, 4, 6, 8, 10, 12, 14, 16, 20 and 24 hours and then analysed as before.

Chromatographic analysis

The column used was a Chiralpak AD CSP (250 x 4.6 mm, 10 µm) connected to a guard column containing similar material (50 x 4.6 mm, 10 µm). The mobile phase consisted of hexane:ethanol (90:10, v/v) containing TFA (0.05% v/v) as modifier, at a flow rate of 1.0 ml/min. The fluorescence detector was set at excitation and emission wavelengths of 288 and 340 nm respectively and UV detection was performed at 254 nm.

Validation of the assay procedure

A stock solution containing (*R,S*)-flurbiprofen (10 mg/100ml), (*R,S*)-4'-hydroxy-flurbiprofen (20mg/100ml) and (*R,S*)-3'-hydroxy-4'-methoxyflurbiprofen (2mg/100ml) was prepared in acetonitrile. Into nine separate 20 ml volumetric flasks were pipetted 0.02, 0.04, 0.1, 0.2, 0.4, 1.0, 2.0, 3.0 and 4.0 ml of the stock solution. The solutions were evaporated gently under nitrogen gas. Blank drug free urine was added to the flasks q.s. 20 ml to give final solutions containing 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 7.5 and 10 μg/ml of each flurbiprofen enantiomer; 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 15.0 and 20 μg/ml of each 4'-hydroxyflurbiprofen enantiomer and 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 1.5, and 2.0 μg/ml of each 3'-hydroxy-4'-methoxyflurbiprofen enantiomer in the respective flasks. Aliquots (1.0 ml) of these standards were transferred into different tubes and stored at -20°C. On each day of analysis one set of these tubes were analysed together with the samples. Ultraviolet detection at 254 nm was used for monitoring flurbiprofen, 4'-hydroxyflurbiprofen and the higher concentrations of 3'-hydroxy-4'-methoxyflurbiprofen (calibration range: 0.1-2.0 μg/ml for each enantiomer). However, fluorescence detection was used for lower concentrations of 3'-hydroxy-4'-methoxy-flurbiprofen (calibration range: 0.01-1.0 μg/ml for each enantiomer) the excitation wavelength was 288 nm and the emission cut-off filter was 340 nm. Calibration curves were constructed by plotting peak area ratios (analyte enantiomer:(*S*)-naproxen) against the concentration of each enantiomer and subjecting the data to linear regression analysis. The concentration of each analyte enantiomer in urine samples were determined by comparing their respective peak area ratios to the calibration curve prepared.

The within day variation of the assay for flurbiprofen, 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen was assessed by analysing six standard samples each of nominal high, medium and low concentrations (refer to Tables 5.2 to 5.4). The precision and accuracy of the assay was determined for each analyte by calculation of the percentage coefficient of variation (standard deviation/mean x 100) and mean percentage difference ([mean-actual concentration]/actual concentration x 100) respectively. The extraction efficiencies for flurbiprofen and the metabolites were estimated by comparing peak areas against theoretical 100 % values obtained by analysis of standard solutions prepared in hexane:ethanol (90:10, v/v), containing an equivalent quantity of analyte on column.

The between day variation of the assay was determined by analysing urine standard samples of the same concentrations for six consecutive days. The precision and accuracy were calculated as described above.

In order to determine whether the analytical procedure could produce accurate data with respect to the enantiomeric composition of flurbiprofen over a wide range of concentrations, a series of "spiked" standards of varying concentration and enantiomeric composition were prepared using the individual flurbiprofen enantiomers. "Total" flurbiprofen concentrations of 0.5, 2.0 and 10 μg/ml were prepared in urine with the following enantiomeric compositions; *R*:*S*; 1:4; 2:3; 3:2 and 4:1 for each concentration. The samples were extracted and analysed in triplicate and the precision and accuracy of the method was determined for each enantiomeric composition at the chosen concentrations as described above.

The applicability of the de-conjugation methodologies for quantitative analysis was assessed by determining the precision following the replicate analysis (n=6) of a volunteer's 4-6 hr post-dose urine collection, hydrolysed using the enzyme plus base approach and the base approach.

5.2.5 Enantiospecific analysis of flurbiprofen and 4′-hydroxyflurbiprofen in serum

Sample preparation

To serum samples (1.0 ml) were added 0.25 μg (50 μl of a 5.0 μg/ml solution in acetonitrile) of (*R*,*S*)-benoxaprofen as internal standard. The samples were then acidified by the addition of hydrochloric acid (1.0 M; 100 μl) and buffered to pH 3.8 with 1.0 ml of phosphate buffer (pH 3.8; 1.0 M). Hexane:ethyl acetate (90:10 v/v; 6 ml) was added and the extraction tubes were then tightly capped and mixed on a test-tube rocker for 20 minutes. Phase separation was achieved by centrifugation for 5 minutes at 1000 g. The organic layer was then separated into a clean glass tube and evaporated under a gentle stream of nitrogen at 40°C on a dry heating block. The residue was then reconstituted in 150 μl mobile phase and 100 μl injected into the chiral HPLC system.

Chromatographic analysis

The column used was a Chiralpak AD CSP (250 x 4.6 mm, 10 μm) connected to a guard column containing similar material (50 x 4.6 mm, 10 μm). The mobile phase consisted of hexane:ethanol (87:13, v/v) containing TFA (0.05% v/v) as modifier, at a flow rate of 1.0ml/min. The fluorescence detector was set at excitation and emission wavelengths of 288 and 340 nm respectively.

Validation of the assay procedure

A stock solution containing (*R,S*)-flurbiprofen (10 mg/100ml) and (*R,S*)-4'-hydroxyflurbiprofen (1.0 mg/100ml) was prepared in acetonitrile. Into nine separate 20 ml volumetric flasks were pipetted 0.04, 0.08, 0.16, 0.24, 0.32, 0.4, 0.8, 1.6 and 2.4 ml of the stock solution. The solutions were evaporated gently under nitrogen gas. drug free serum was added to the flasks q.s. 20 ml to give final solutions containing 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 4.0 and 6 μg/ml of each flurbiprofen enantiomer and 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4 and 0.6 μg/ml of each 4'-hydroxyflurbiprofen enantiomer in the respective flasks. Aliquots (1.0ml) of these standards were transferred into different tubes and stored at -20°C. On each day of analysis one set of these tubes were analysed together with the samples. Calibration curves were constructed by plotting peak height ratios (analyte enantiomer : first eluting enantiomer of benoxaprofen) against the concentration of each enantiomer and subjecting the data to linear regression analysis. The concentration of each analyte enantiomer in serum samples were determined by comparing their respective peak height ratios to the calibration curve prepared.

The within day variation of the assay for flurbiprofen and 4'-hydroxyflurbiprofen was assessed by analysing six standard samples each of nominal high, medium and low concentrations (refer to Tables 5.8 and 5.9). The precision and accuracy of the assay was determined for each analyte by calculation of the percentage coefficient of variation (standard deviation/mean x 100) and mean percentage difference ([mean-actual concentration]/actual concentration x 100) respectively. The extraction efficiencies for flurbiprofen and the metabolites were estimated by comparing peak areas against theoretical 100 % values obtained by analysis of standard solutions prepared in hexane:ethanol (87:13 v/v), containing an equivalent quantity of analyte on column.

The between day variation of the assay was determined by analysing serum standard samples of the same concentrations for six consecutive days. The precision and accuracy were calculated as described above.

In order to determine whether the analytical procedure could produce accurate data with respect to the enantiomeric composition of flurbiprofen over a wide range of concentrations, a series of "spiked" standards of varying concentration and enantiomeric composition were prepared using the individual flurbiprofen enantiomers. "Total" flurbiprofen concentrations of 0.5, 2.0 and 6 μg/ml were prepared in serum with the following enantiomeric compositions; *R*:*S*; 1:4; 2:3; 3:2 and 4:1 for each concentration. The samples were extracted and analysed in triplicate and the precision and accuracy of the method was determined for each enantiomeric composition at the chosen concentrations as described above.

5.2.6 Preliminary urinary excretion studies following the administration of (*R*,*S*)-flurbiprofen, (*S*)-flurbiprofen or (*R*)-flurbiprofen

Three healthy male volunteers (volunteers A, B and C: aged 48, 35 and 28 years, weight 68, 81 and 70 kg respectively) were given a single oral dose of racemic flurbiprofen (1 x 100 mg Froben® tablet) with 150 ml water following an overnight fast. Volunteer A was also administered 50 mg (*R*)-flurbiprofen and 50 mg (*S*)-flurbiprofen with a washout interval between each dose of at least 2 weeks.

A blank, drug-free, urine sample was obtained prior to drug administration and sequential samples were collected at two hourly intervals up to 12 hours post dosing, together with a 12-24 h sample. The individual urine volumes were recorded and the samples analysed using the procedures described in section 5.2.4.

5.2.7 Preliminary serum study following the administration of (*R*,*S*)-flurbiprofen

Blood samples were also taken from volunteer A following the administration of the 100mg racemic dose. A venous blood sample (10 ml) was collected prior to flurbiprofen dosing and at 0.25, 0.50, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10 and 24 h post administration. Blood drawn by a plastic syringe was immediately transferred into glass tubes and allowed to clot at room temperature for two hours. Serum was separated by

centrifugation (10 min at 2000 g) and analysed using the procedure described in section 5.2.5.

5.3. Results and Discussion

5.3.1 Enantiospecific analysis of flurbiprofen and its metabolites in urine

The achievement of the desired chromatographic separation profile is only an initial preliminary step in the development of a quantitative enantiospecific urine assay method. Some of the critical, and often more challenging, issues that need to be addressed include selection of an appropriate detection method, the choice of an internal standard, the development of a suitable sample extraction procedure and adaptation of the methodology for the quantitative analysis of drug conjugates and metabolite conjugates.

The presence of the drug and metabolites at widely different concentration ranges in urine, make it almost impossible to use a single detector with acceptable sensitivity for the analysis of all the analytes. However, the fluorescent nature of flurbiprofen and its metabolites allow for the use of tandem fluorescence and UV detectors to overcome this problem. The fluorescence detector was set at excitation and emission wavelengths of 288 nm and 340 nm respectively to provide optimum response for the detection of 3'-hydroxy-4'-methoxyflurbiprofen, the enantiomers of this analyte are present at the lowest concentrations in urine samples and being the later eluting-peaks on the Chiralpak AD CSP exhibit the poorer peak shapes in terms of band broadening. The UV detector was used at 254 nm and was of sufficient sensitivity to allow for the simultaneous analysis of the enantiomers of flurbiprofen, 4'-hydroxyflurbiprofen and also higher concentrations of 3'-hydroxy-4'-methoxyflurbiprofen (> 0.1 μg/ml for each enantiomer in urine).

Selection of an appropriate internal standard for chiral-phase bioanalysis is often difficult since CSPs tend to show poor separation characteristics for structurally similar compounds and furthermore in this case the choice is restricted by the need that candidates possess acceptable fluorescence and UV-absorbing properties under the experimental conditions employed. Therefore the compounds investigated as potential internal standards were other highly conjugated 2-arylpropionic acids and of these only

(*S*)-naproxen met the desired criteria. As shown in Figure 5.2, (*S*)-naproxen having a retention time of 10.1 minutes was baseline resolved between the peaks of (*S*)-flurbiprofen and (*R*)-4'-hydroxyflurbiprofen and could be readily detected using both detection systems.

The operation of the Chiralpak AD stationary phase under normal-phase conditions makes it not ideally suited for applications in bioanalysis since most potentially interfering compounds derived from the biological matrix are polar and can be strongly retained on the chromatographic support. The presence of these contaminants may cause problems in analyte retention, resolution and column stability (Ducharme *et al.*, 1996; Hutt and Patel, 1998); and therefore it is essential that extremely clean extracts are obtained. Initial 'scouting' examinations of various extraction solvents for the isolation of flurbiprofen and its metabolites from urine were performed with the use of an achiral reversed-phase separation method rather than the CSP. From these preliminary studies it was deemed that a hexane:isopropanol (95:5 v/v) mixture was the most appropriate for evaluation and determination of extraction efficiencies on the chiral stationary phase. Typical chromatograms obtained using the Chiralpak AD CSP for extracts from a blank urine sample and a "spiked" urine standard are shown in Figure 5.2. The stable baseline observed after the initial solvent front for the extract of blank urine illustrates the effectiveness of the extraction method as a clean-up process; and as expected the chromatogram obtained using fluorescence detection gave a relatively "cleaner" appearance due to its greater selectivity.

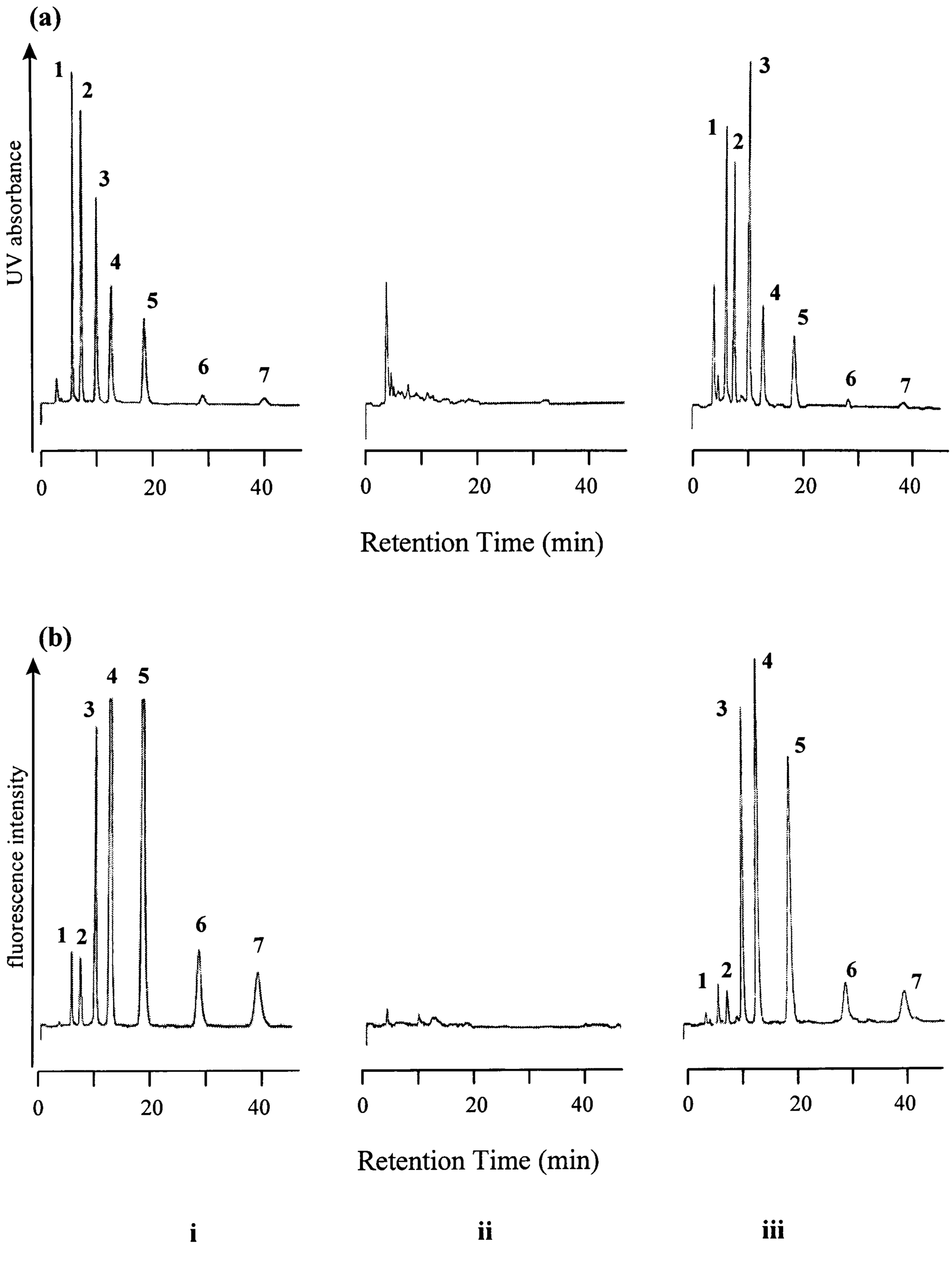


Figure 5.2: Chiral-phase chromatograms of (i) reference standards, extracts of (ii) blank drug free urine and (iii) "spiked" urine; using (a) UV detection and (b) fluorescence detection. Peaks : 1, (*R*)-flurbiprofen; 2, (*S*)-flurbiprofen; 3, (*S*)-naproxen (I.S.); 4, (*R*)-4'-hydroxyflurbiprofen; 5, (*S*)-4'-hydroxyflurbiprofen; 6, (*R*)-3'-hydroxy-4'-methoxyflurbiprofen and 7, (*S*)-3'-hydroxy-4'-methoxyflurbiprofen [Mobile phase hexane : ethanol (90:10 v/v) containing trifluoroacetic acid (0.05% v/v); Flow rate, 1.0ml/min].

Extraction efficiencies were determined at three different concentrations for each of the analytes and the results are presented in Table 5.1. The calculated percentages are based on theoretical 100 % values determined by analysis of standards containing equivalent quantities of the analytes. Essentially by employing this extraction system, high recovery of the polar metabolites is sacrificed for the maintenance of "contaminant-free" extracts.

Table 5.1: Liquid-liquid extraction efficiencies for the enantiomer of flurbiprofen and its metabolites from urine using a hexane:isopropanol (95:5 v/v) mixture (mean ± s.d.; n=6).

Analyte	Enantiomeric Concentration (mcg/ml)	Extraction Efficiency (%)	
		R-isomer	*S*-isomer
Flurbiprofen	0.10	89.5 ± 6.8	88.9 ± 5.9
	0.50	89.2 ± 2.5	89.0 ± 2.7
	5.00	86.8 ± 1.9	86.5 ± 1.6
4'-Hydroxyflurbiprofen	0.20	74.5 ± 4.8	74.2 ± 3.8
	1.00	75.2 ± 2.8	74.9 ± 2.8
	10.0	75.4 ± 1.1	75.1 ± 1.3
3'-Hydroxy-4'-methoxyflurbiprofen	0.02	78.3 ± 6.8	77.8 ± 7.7
	0.10	79.1 ± 4.8	78.8 ± 4.2
	1.00	77.4 ± 2.1	77.1 ± 2.2

Optimisation of enzymatic hydrolysis conditions

Previous assay methods have quantified the conjugates of flurbiprofen, and its metabolites, by using either base (Adams *et al.*, 1987) or acid hydrolysis (Knadler and Hall, 1989). However, in addition to acyl glucuronidation, the metabolites of flurbiprofen undergo phenolic conjugation to form ether glucuronides and sulphate conjugates and the disadvantage of the above methodologies is that they do not differentiate between conjugation at the different sites. Therefore, we have employed a enzymatic-base hydrolysis combination method, in addition to a base hydrolysis procedure that allows for the quantification of both acyl- and phenolic-conjugation of

the flurbiprofen metabolites. Phenolic conjugates tend to be relatively stable and so mild basic conditions are suitable only for the liberation of the free acids from their ester conjugates, including any isomers that may have arisen from intramolecular rearrangement (acyl migration). A partially purified form of β-glucuronidase, namely β-glucuronidase (EC 3.2.1.31) type H-5, was selected for enzymatic hydrolysis as it also contains sulphatase activity and so will hydrolyse both phenolic- and 1-*O*-acyl-glucuronides in addition the sulphate conjugates. But it is worth noting that the glucuronides derived via acyl migration are not susceptible to enzymatic hydrolysis (Caldwell *et al.*, 1983).

The enzymatic hydrolysis of the enantiomers of flurbiprofen and its metabolites was optimised with respect to the quantity of β-glucuronidase and the incubation time required. The influence of quantity of β-glucuronidase on hydrolysis was investigated by incubating volunteer urine samples with 500, 1000, 2000, 4000 and 6000 units of β-glucuronidase for a period of 4 hours and 24 hours (Figure 5.3). Increasing the amount of β-glucuronidase beyond 1000 units per incubate appeared to have minimal influence on the hydrolysis of any the analytes, whether it be for the 4 hr or 24 hr incubations. The effect of increasing the incubation period from 4 hours to 24 hours, with a 1000 units of β-glucuronidase per incubate, was to improve the yields for the enantiomers of 3'-hydroxy-4'-methoxyflurbiprofen by approximately 15 %, the increase in yields for the enantiomers of flurbiprofen and 4'-hydroxyflurbiprofen were not as significant and tended to be less than 5% (Figure 5.3). Evidently, the hydrolysis reaction is not complete within 4 hours and so its time course was followed, using 1000 units of β-glucuronidase per incubate, at regular intervals over a 24 hour period to establish the optimal incubation time. The data presented in Figure 5.4 shows that hydrolysis of flurbiprofen conjugates and 4'-hydroxyflurbiprofen conjugates was relatively rapid and essentially complete after 6 hours, however the reaction appeared to have slower kinetics for 3'-hydroxy-4'-methoxyflurbiprofen and complete liberation of the aglycone is achieved after 14 hours. The enantiomeric composition of all three analytes remained essentially constant throughout the experiment, indicating that there was no stereoselective hydrolysis under the experimental conditions employed. (Figure 5.4). Upon this basis, hydrolysis of urine samples with a 1000 units of β-glucuronidase for a duration of 16 hours, which is a convenient time period for over-night incubation, was deemed most

appropriated for application in the urine assay method. The base-hydrolysis procedure is essentially that previously characterised and employed in the enantiospecific urine assay methods for ibuprofen and its metabolites, see Chapter 2.

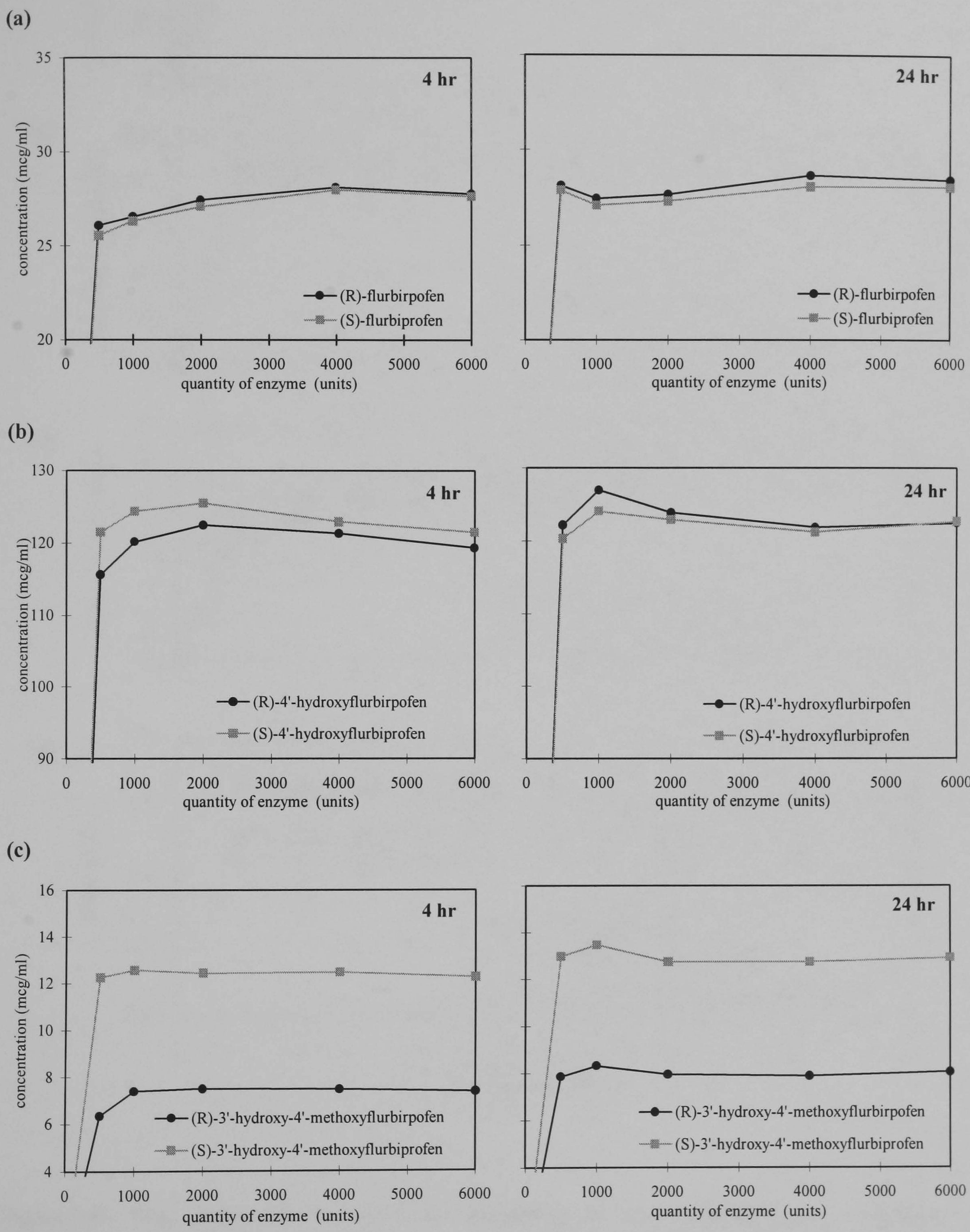


Figure 5.3: Effect of quantity of β-glucuronidase on the hydrolysis of conjugates of the enantiomers of (a) flurbiprofen, (b) 4'-hydroxyflurbiprofen and (c) 3'-hydroxy-4'-methoxy-flurbiprofen over 4 hour and 24 hour incubation periods [Sample: 4-6 hr urine collection following oral administration of 100 mg racemic flurbiprofen to volunteer C; n=2].

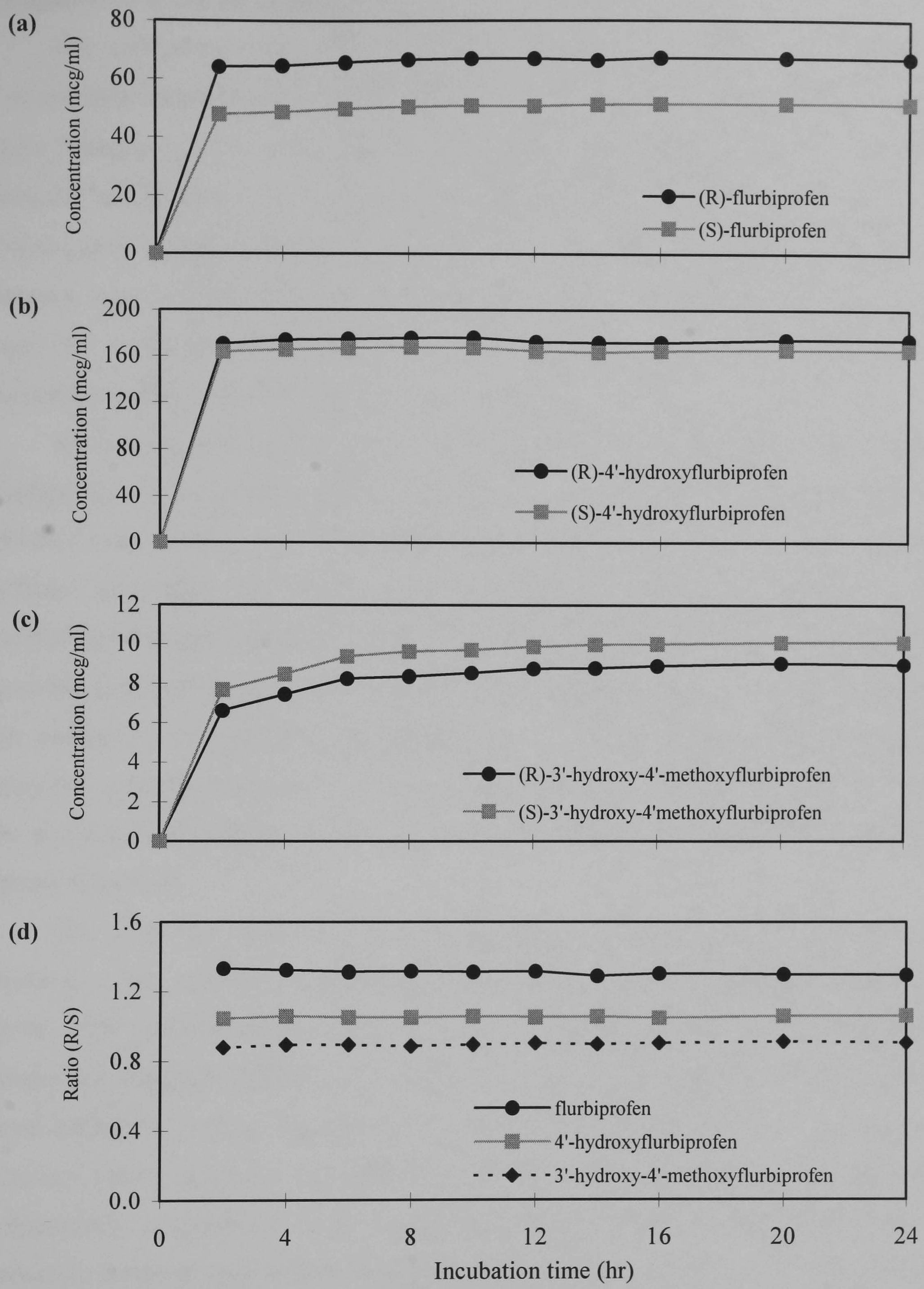


Figure 5.4: Time course of hydrolysis for conjugates of (a) flurbiprofen, (b) 4'-hydroxy-flurbiprofen and (c) 3'-hydroxy-4'-methoxyflurbiprofen by β-glucuronidase and (d) the corresponding R/S ratios [Sample: 4-6 hr urine collection following oral administration of 100 mg racemic flurbiprofen to volunteer A; n=2].

Validation of urine assay procedure

The calibration curves for the analysis of the enantiomers of flurbiprofen, 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen isolated from urine, were linear between 0.05-10 μg/ml, 0.1-20 μg/ml and 0.1-2.0 μg/ml respectively using UV detection and are shown in Figure 5.5. The optimisation of the fluorescence detector for 3'-hydroxy-4'-methoxyflurbiprofen allowed for the sensitive analysis of its enantiomers between 0.01-1.0 μg/ml, a typical calibration curve is shown in Figure 5.6. Linear regression analysis of the calibration curves for all the analytes routinely gave correlation coefficients better than 0.998.

Analytical precision and accuracy were established by adding known quantities of flurbiprofen, 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen at three different concentrations to urine and analysing aliquots on a single day (n=6) and on six different assay days, and the results are presented in Tables 5.2, 5.3 and 5.4. The coefficient of variation of the analytical method for within- and between-day studies was generally less than 10 % for all the analytes, indicating the precision of the assay at all the concentrations examined was greater than 90 %. The mean difference of the analytical method for within- and between-day studies was also less than 10 % for all the analytes, indicating the accuracy of the assay at all the concentrations examined was greater than 90 %.

The assay was further validated by analysing a series of samples "spiked" with mixtures of the individual enantiomers of flurbiprofen at three different concentration levels. This validation approach is necessary as biological samples from pharmaco-kinetic and metabolic studies will contain non-racemic mixtures of enantiomers due to stereoselectivity in drug metabolism and disposition (Hutt, 1991). The precision and accuracy values calculated for these analyses are shown in Table 5.5. The measured enantiomeric compositions were in good agreement with the expected values and the variation involved was within acceptable limits at all three "total" concentrations examined. These results indicate that racemization is not occurring during sample manipulation and that the concentration of flurbiprofen does not appear to affect the measured enantiomeric composition. Due to the unavailability of sufficient quantities of the individual enantiomers of the metabolites, this validation approach was not performed for 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen.

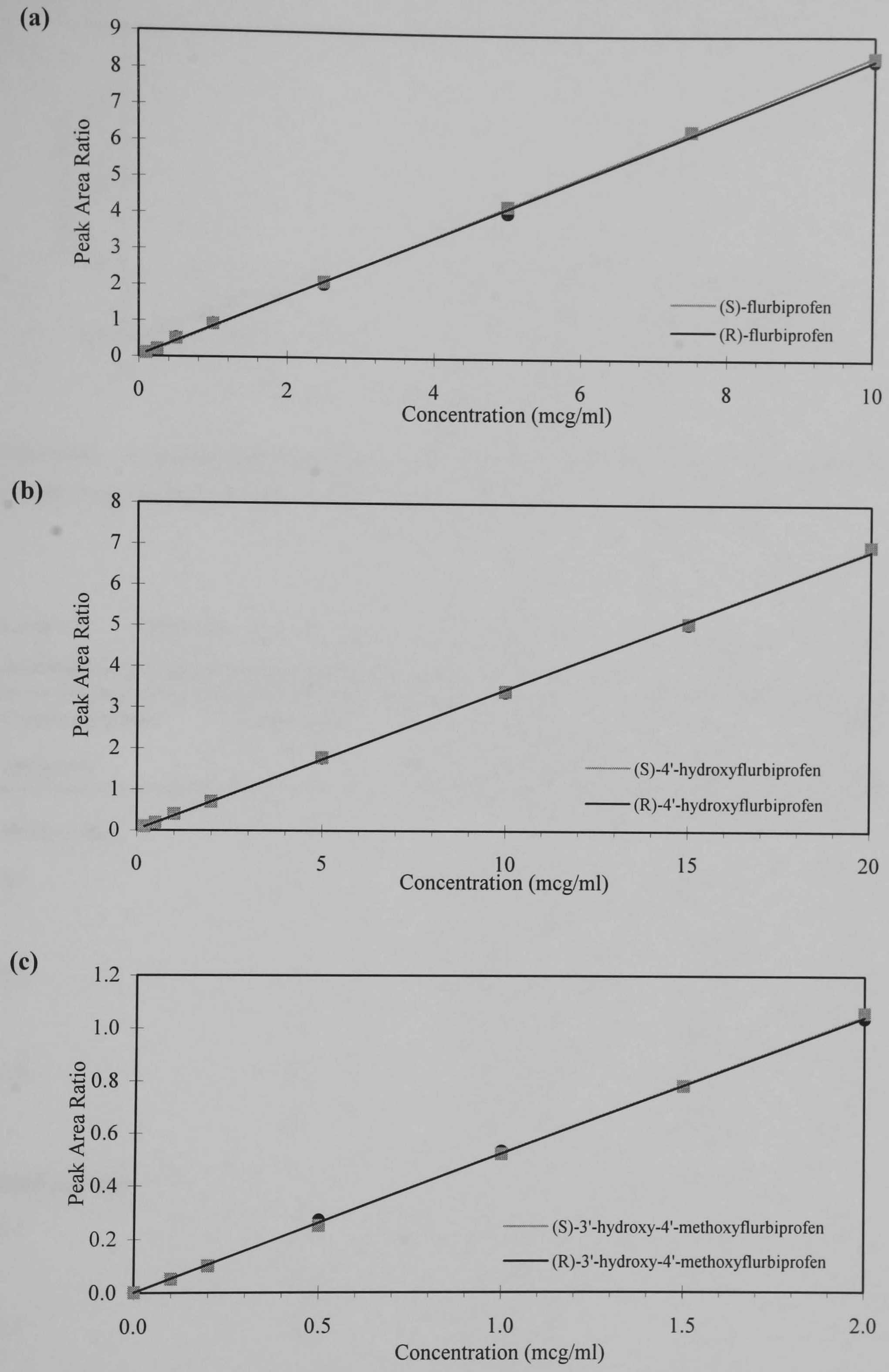


Figure 5.5: Typical calibration curves prepared for the quantification of the enantiomers of (a) flurbiprofen, (b) 4'-hydroxyflurbiprofen and (c) 3'-hydroxy-4'-methoxyflurbiprofen in urine samples using UV detection.

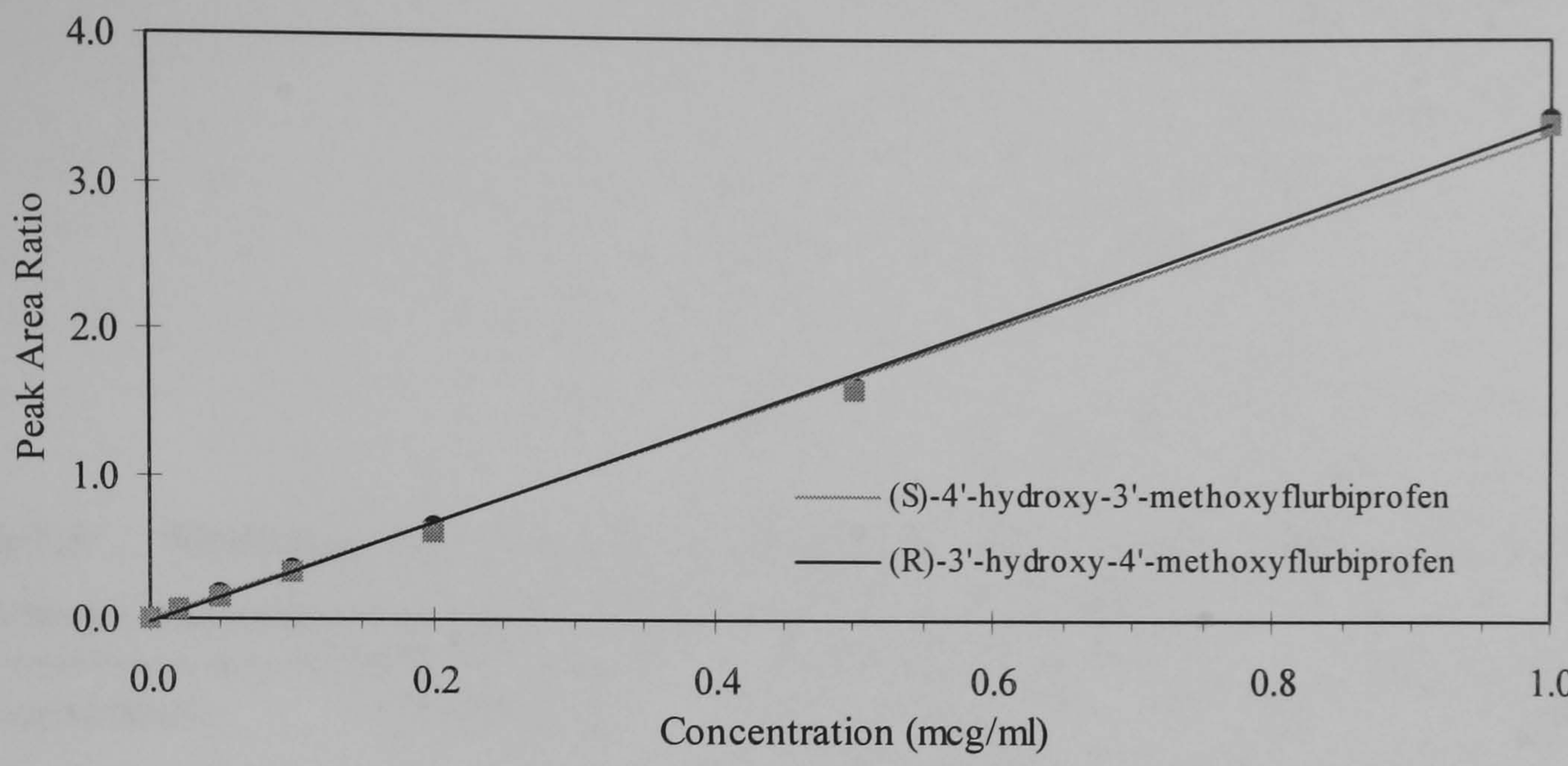


Figure 5.6: A typical calibration curve prepared for the quantification of the enantiomers of 3'-hydroxy-4'-methoxyflurbiprofen in urine samples using fluorescence detection.

Table 5.2: Within-day and between-day analytical variation and accuracy of flurbiprofen enantiomers in "spiked" urine samples (mean ± s.d.; n=6) *.

Concentration (mcg/ml)	Enantiomer	Concentration determined (mcg/ml)	C.V. (%)	M.D. (%)
Within-day				
0.1	*R*	0.108 ± 0.008	7.47	8.0
	S	0.103 ± 0.007	6.39	3.0
0.5	*R*	0.47 ± 0.02	4.05	-6.0
	S	0.50 ± 0.02	3.49	0.0
5.0	*R*	5.04 ± 0.15	2.97	0.8
	S	5.03 ± 0.15	2.88	0.6
Between-day				
0.1	*R*	0.103 ± 0.011	10.51	3.0
	S	0.098 ± 0.011	11.61	-2.0
0.5	*R*	0.49 ± 0.01	2.10	-2.0
	S	0.49 ± 0.02	3.20	-2.0
5.0	*R*	5.01 ± 0.13	2.64	0.2
	S	4.98 ± 0.10	2.02	-0.4

** C.V. = coefficient of variation; M.D. = mean difference.*

Table 5.3: Within-day and between-day analytical variation and accuracy of 4'-hydroxy-flurbiprofen enantiomers in "spiked" urine samples (mean ± s.d.; n=6) *.

Concentration (mcg/ml)	Enantiomer	Concentration determined (mcg/ml)	C.V. (%)	M.D. (%)
Within-day				
0.2	*R*	0.211 ± 0.009	4.25	5.5
	S	0.203 ± 0.008	3.98	1.5
1.0	*R*	1.05 ± 0.03	2.86	5.0
	S	1.04 ± 0.03	2.68	4.0
10.0	*R*	10.10 ± 0.29	2.84	1.0
	S	10.10 ± 0.29	2.90	1.0
Between-day				
0.2	*R*	0.207 ± 0.008	3.89	3.5
	S	0.201 ± 0.009	4.35	0.5
1.0	*R*	1.02 ± 0.05	4.40	2.0
	S	0.99 ± 0.06	5.86	-1.0
10.0	*R*	9.88 ± 0.41	4.15	-1.2
	S	9.87 ± 0.45	4.60	-1.3

* *C.V. = coefficient of variation; M.D. = mean difference.*

Table 5.4: Within-day and between-day analytical variation and accuracy of 3'-hydroxy-4'-methoxyflurbiprofen enantiomers in "spiked" urine samples (mean ± s.d.; n=6) *.

Concentration (mcg/ml)	Enantiomer	Concentration determined (mcg/ml)	C.V. (%)	M.D. (%)
Within-day				
0.02	*R*	0.020 ± 0.001	6.32	0.0
	S	0.019 ± 0.001	6.10	-5.0
0.1	*R*	0.099 ± 0.003	2.63	-1.0
	S	0.098 ± 0.002	2.54	-2.0
1.0	*R*	1.05 ± 0.04	3.86	5.0
	S	1.04 ± 0.04	3.80	4.0
Between-day				
0.02	*R*	0.021 ± 0.002	8.03	5.0
	S	0.020 ± 0.002	11.91	0.0
0.1	*R*	0.098 ± 0.008	7.71	-2.0
	S	0.096 ± 0.007	7.62	-4.0
1.0	*R*	1.03 ± 0.04	4.20	3.0
	S	1.02 ± 0.05	4.52	2.0

* *C.V. = coefficient of variation; M.D. = mean difference.*

Table 5.5: Precision and accuracy data for the determination of a series of different enantiomeric compositions of flurbiprofen in urine at three different "total" concentrations (mean ± s.d.; n=3)*.

Enantiomer	Theoretical concentration(mcg/ml)	Measured concentration(mcg/ml)	C.V. (%)	M.D. (%)
Low concentration (0.5 mcg/ml)				
R	0.1	0.111 ± 0.009	7.64	11.3
S	0.4	0.431 ± 0.005	1.16	7.8
R	0.2	0.209 ± 0.006	2.91	4.5
S	0.3	0.318 ± 0.007	2.10	5.9
R	0.3	0.305 ± 0.012	4.10	1.7
S	0.2	0.217 ± 0.005	2.08	8.3
R	0.4	0.410 ± 0.014	3.33	2.6
S	0.1	0.109 ± 0.002	1.41	8.7
Medium concentration (2.0 mcg/ml)				
R	0.4	0.402 ± 0.008	2.07	0.4
S	1.6	1.595 ± 0.017	1.05	-0.3
R	0.8	0.800 ± 0.012	1.52	0.0
S	1.2	1.188 ± 0.019	1.61	-1.0
R	1.2	1.190 ± 0.006	0.46	-0.9
S	0.8	0.788 ± 0.015	1.88	-1.5
R	1.6	1.610 ± 0.001	0.04	0.6
S	0.4	0.390 ± 0.007	1.68	-2.5
High concentration (10.0 mcg/ml)				
R	2.0	2.17 ± 0.053	2.44	8.6
S	8.0	7.89 ± 0.193	2.44	-1.3
R	4.0	3.94 ± 0.100	2.54	-1.4
S	6.0	5.99 ± 0.151	2.52	-0.1
R	6.0	6.02 ± 0.072	1.20	0.3
S	4.0	4.01 ± 0.031	0.77	0.3
R	8.0	7.94 ± 0.045	0.56	-0.7
S	2.0	2.03 ± 0.019	0.93	1.3

* *C.V. = coefficient of variation; M.D. = mean difference.*

The methodologies established for the determination of the conjugates of flurbiprofen and its metabolites were assessed for their suitability by determining the precision following the replicate analysis (n=6 in each case) of a volunteer's urine sample after enzymatic plus base hydrolysis and base hydrolysis alone. As expressed in Table 5.6, the coefficient of variation was generally less than 6 % for all the analytes following either de-conjugation procedure and thus indicated the utility of the approaches for quantitative analysis. It can also be observed from these validation experiments, the close correlation of the determined concentrations for flurbiprofen enantiomers between the two different hydrolysis methods, which is indicative of the fact that flurbiprofen cannot undergo phenolic conjugation.

Table 5.6: Precision data for the determination of the enantiomers of flurbiprofen and its metabolites following enzymatic plus base hydrolysis and base hydrolysis (mean ± s.d.; n=6) *.

Analyte	Enantiomer	Concentration determined (mcg/ml)	C.V. (%)
Enzymatic + base hydrolysis			
Flurbiprofen	*R*	69.9 ± 1.9	2.66
	S	56.0 ± 1.5	2.70
4'-Hydroxyflurbiprofen	*R*	126.5 ± 4.3	3.40
	S	109.2 ± 3.6	3.31
3'-Hydroxy-4'-methoxyflurbiprofen	*R*	7.33 ± 0.11	1.55
	S	8.72 ± 0.15	1.72
Base hydrolysis			
Flurbiprofen	*R*	68.3 ± 1.5	2.22
	S	54.8 ± 1.3	2.28
4'-Hydroxyflurbiprofen	*R*	124.0 ± 6.9	5.55
	S	108.8 ± 6.5	5.93
3'-Hydroxy-4'-methoxyflurbiprofen	*R*	0.83 ± 0.01	1.37
	S	1.33 ± 0.03	2.15

** C.V. = coefficient of variation.*

[Sample: 4-6 hr urine collection following oral administration of 100 mg racemic flurbiprofen to volunteer B].

5.3.2 Enantiospecific analysis of flurbiprofen and 4′-hydroxyflurbiprofen in serum

A preliminary investigation of volunteer serum samples using the methodology adopted for the analysis of urine samples indicated that the approach would require modification, in terms of the detection method, mobile phase composition and choice of internal standard, for application in serum analysis.

Initial studies indicated that, under the optimised conditions for fluorescence detection, the enantiomers of 3'-hydroxy-4'-methoxyflurbiprofen could not be detected in serum samples of a volunteer following administration of racemic flurbiprofen, these observations are in accordance with the findings of Adams *et al.* (1987) and Knadler *et al.* (1992a). The development of the serum assay was consequently limited to the quantitative analysis of the enantiomers of flurbiprofen and 4'-hydroxyflurbiprofen. It was deemed that the use of a single detection method would be sufficient for this purpose and so the fluorescence detector was employed and set at an optimum sensitivity for 4'-hydroxyflurbiprofen, which was expected to be present at lower concentrations than the parent compound in serum. It is worth noting that 4'-hydroxy-flurbiprofen exhibited similar fluorescence excitation and emission maxima to those of 3'-hydroxy-4'-methoxyflurbiprofen.

In addition, close examination of the chromatograms obtained following analysis of extracts of volunteer serum post drug administration revealed the presence of a slight shoulder on the peak of (*S*)-4'-hydroxyflurbiprofen (Figure 5.7a). As the "shoulder" was not observed in chromatograms obtained using "spiked" serum standards, and extracts of blank serum did not show additional peaks in this region of the chromatogram, it was thought that the "shoulder" could be due to co-eluting drug-related material. Also if the peak is due to drug-related material, then as the drug is chiral it is highly likely that the unknown is also chiral and that two, rather than one, additional peaks may be present. It was therefore necessary to adjust the mobile phase composition in an attempt to separate the interfering component from (*S*)-4'-hydroxyflurbiprofen. Increasing the ethanol content of the mobile phase from 10 % to 13 % resulted in the separation of the two components with the additional peak eluting just after the tail of the (*S*)-4'-hydroxy-flurbiprofen peak (Figure 5.7b). Examination of hydrolysed and non-hydrolysed volunteer urine samples under these chromatographic conditions did not unmask a

similar peak after the peak of the (*S*)-4'-hydroxy metabolite, suggesting that this particular product was not detectable in urine.

A consequence of the alteration in the mobile phase composition was partial coalescence between the peaks due to (*R*)-4'-hydroxyflurbiprofen and (*S*)-naproxen, the internal standard (Figure 5.7b); therefore it was necessary to find an alternative fluorescent internal standard for application in the serum assay. Racemic benoxaprofen displayed suitable chromatographic characteristics with baseline resolution of its enantiomers and elution after (*S*)-4'-hydroxyflurbiprofen (Figure 5.8a). Either enantiomer of benoxaprofen could have been applied for quantitative analysis thus the choice of the first eluting enantiomer as the internal standard was an arbitrary decision. Due to the non-availability of the individual enantiomers the elution order is unknown. However, previously it has been shown by Booth and Wainer (1996) that the elution order for a series of 2-arylpropionic acids, including flurbiprofen and benoxaprofen, was always the *R*-enantiomer before the *S*-antipode on the amylose tris (3,5-dimethylphenyl-carbamate) CSP and so a fair assumption would be that the (*R*)-benoxaprofen elutes prior to (*S*)-benoxaprofen.

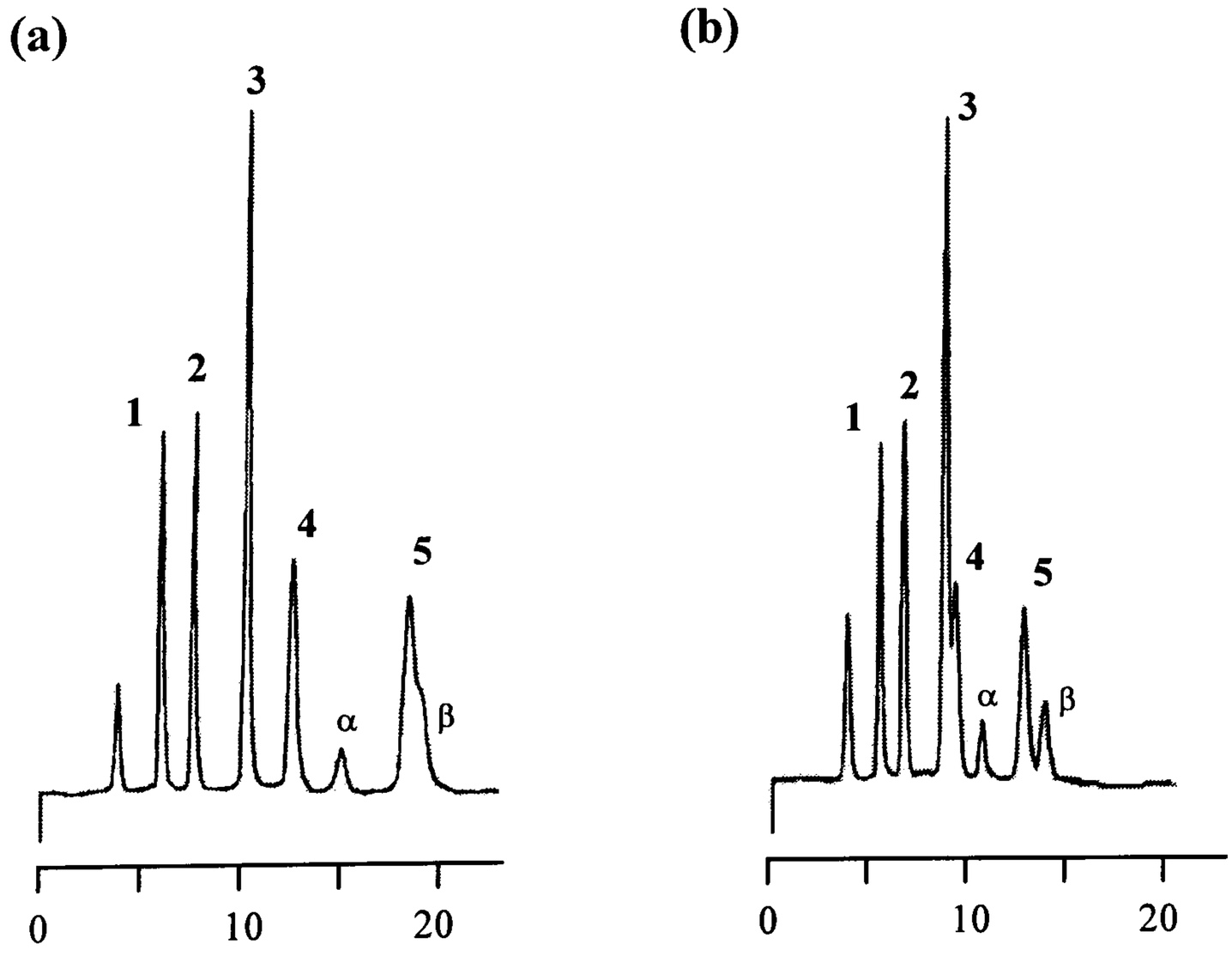


Figure 5.7: Chiral-phase chromatograms of extracts from a serum sample (8 hr) obtained from a volunteer following the oral administration of 100mg racemic flurbiprofen using a mobile phase composition of (a) hexane:ethanol (90:10, v/v) with TFA (0.05%) and (b) hexane:ethanol (87:13 v/v) with TFA (0.05%) Peaks : 1, (*R*)-flurbiprofen; 2, (*S*)-flurbiprofen; 3, (*S*)-naproxen (I.S.); 4, (*R*)-4'-hydroxyflurbiprofen; 5, (*S*)-4'-hydroxyflurbiprofen; α and β, additional unknown peaks.

As with the urine assay, the selection of the most appropriate extraction solvent for the isolation of flurbiprofen and the 4'-hydroxy metabolite from serum was based on "scouting" experiments performed using an achiral reversed-phase column. Employment of the same extraction solvent as used for urine samples was found to be unsuitable as it resulted in the co-extraction of a large amount of serum pigments. A hexane:ethyl acetate (90:10 v/v) mixture displayed more suitable characteristics as it gave clean extracts and good recoveries. Typical chromatograms obtained using the Chiralpak AD CSP for extracts from a blank serum sample and a "spiked" serum standard are shown in Figure 5.8 and illustrate the absence of interfering peaks at the retention times of the analytes and internal standard. Extraction efficiencies, calculated by comparison of peak areas for extracted serum standards against theoretical 100 % values, for the enantiomers of flurbiprofen and 4'-hydroxyflurbiprofen were generally better than 85 % at the concentration levels investigated (Table 5.7).

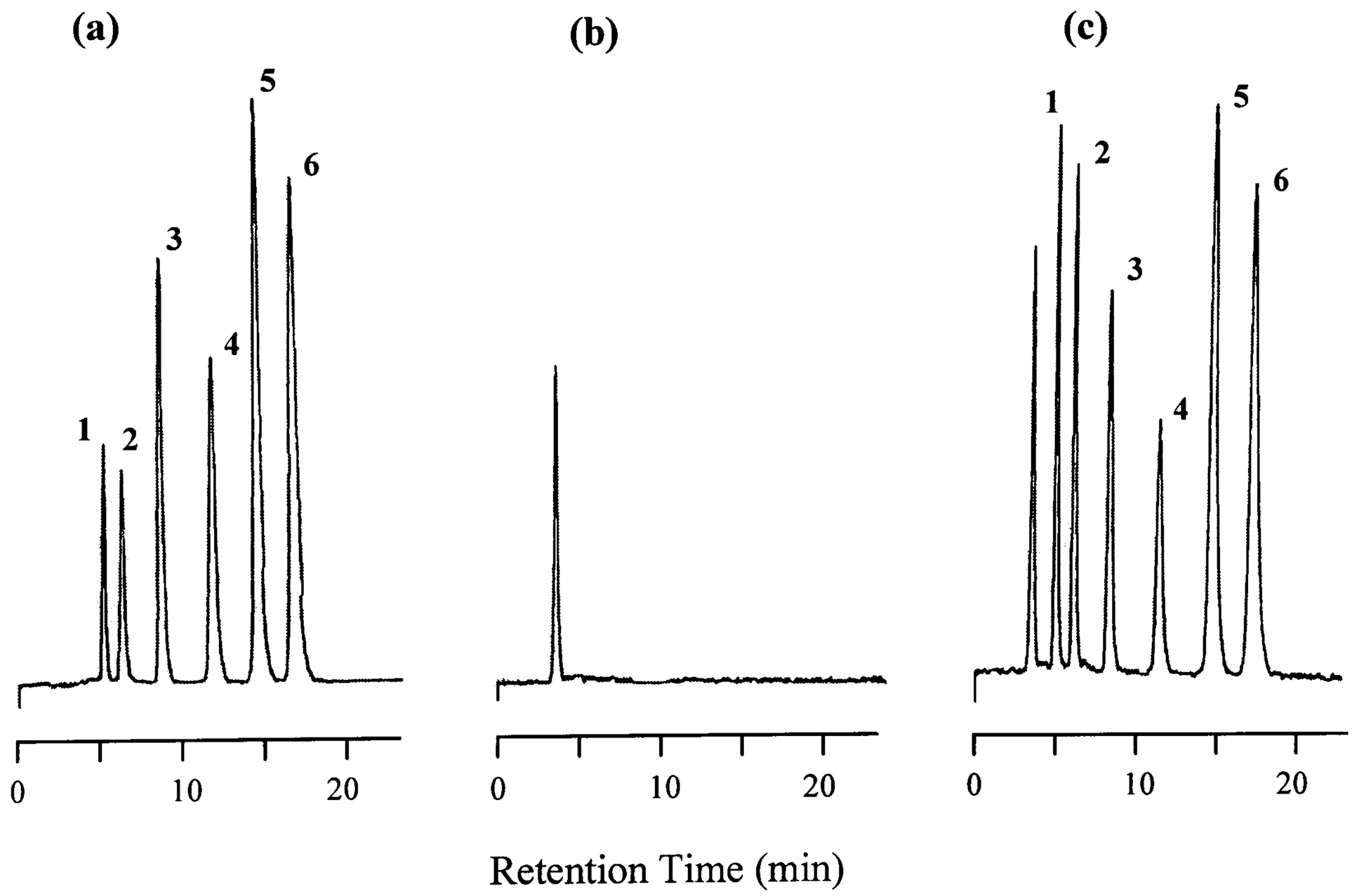


Figure 5.8: Chiral-phase chromatograms of (a) reference standards, extracts of (b) blank serum and (c) "spiked" serum. Peaks : 1, (*R*)-flurbiprofen; 2, (*S*)-flurbiprofen; 3, (*R*)-4'-hydroxy-flurbiprofen; 4= (*S*)-4'-hydroxyflurbiprofen and 5 and 6, enantiomers of benoxaprofen (I.S.) [Mobile phase hexane : ethanol (87:13 v/v) containing TFA (0.05% v/v); Flow rate, 1.0ml/min].

Table 5.7: Liquid-liquid extraction efficiencies for flurbiprofen and 4'-hydroxyflurbiprofen enantiomers from serum using a hexane:ethyl acetate (90:10 v/v) mixture (mean ± s.d.; n=6).

Analyte	Enantiomeric Concentration (mcg/ml)	Extraction Efficiency (%)	
		R-isomer	*S*-isomer
Flurbiprofen	0.20	90.4 ± 10.8	89.3 ± 11.2
	1.00	92.5 ± 5.4	91.9 ± 5.7
	5.00	91.4 ± 2.7	91.0 ± 1.8
4'-Hydroxyflurbiprofen	0.02	85.4 ± 8.8	86.0 ± 9.2
	0.10	86.4 ± 6.3	85.9 ± 5.8
	0.50	87.4 ± 1.6	87.1 ± 1.6

Validation of serum assay procedure

Quantitative analysis of flurbiprofen and 4'-hydroxyflurbiprofen in serum was performed by determining peak-height ratios (analyte : internal standard) instead of peak-area ratios as peak height measurements are less likely to be influenced by the presence of slight overlap between neighbouring peaks, which may be an issue in the chromatographic analysis of volunteer serum extracts due to the exposure of the additional peak at the tail of the (*S*)-4'-hydroxyflurbiprofen peak.

Single enantiomer calibration curves for flurbiprofen (range: 0.1-6.0 μg/ml) and 4'-hydroxyflurbiprofen (range: 0.01-0.6 μg/ml) typically yielded correlation coefficients greater than 0.997 and are shown in Figure 5.9. The within-day and between-day precision and accuracy values for the enantiomers of flurbiprofen and 4'-hydroxy-flurbiprofen, as presented in Tables 5.8 and 5.9, are generally good with coefficient of variations and mean differences less than 6 % at the different concentrations examined Additional validation using non-racemic mixtures of the individual enantiomers of flurbiprofen at various concentrations is shown in Table 5.10. There was good correlation between measured and theoretical enantiomeric compositions and the variability was limited to less than 7%.

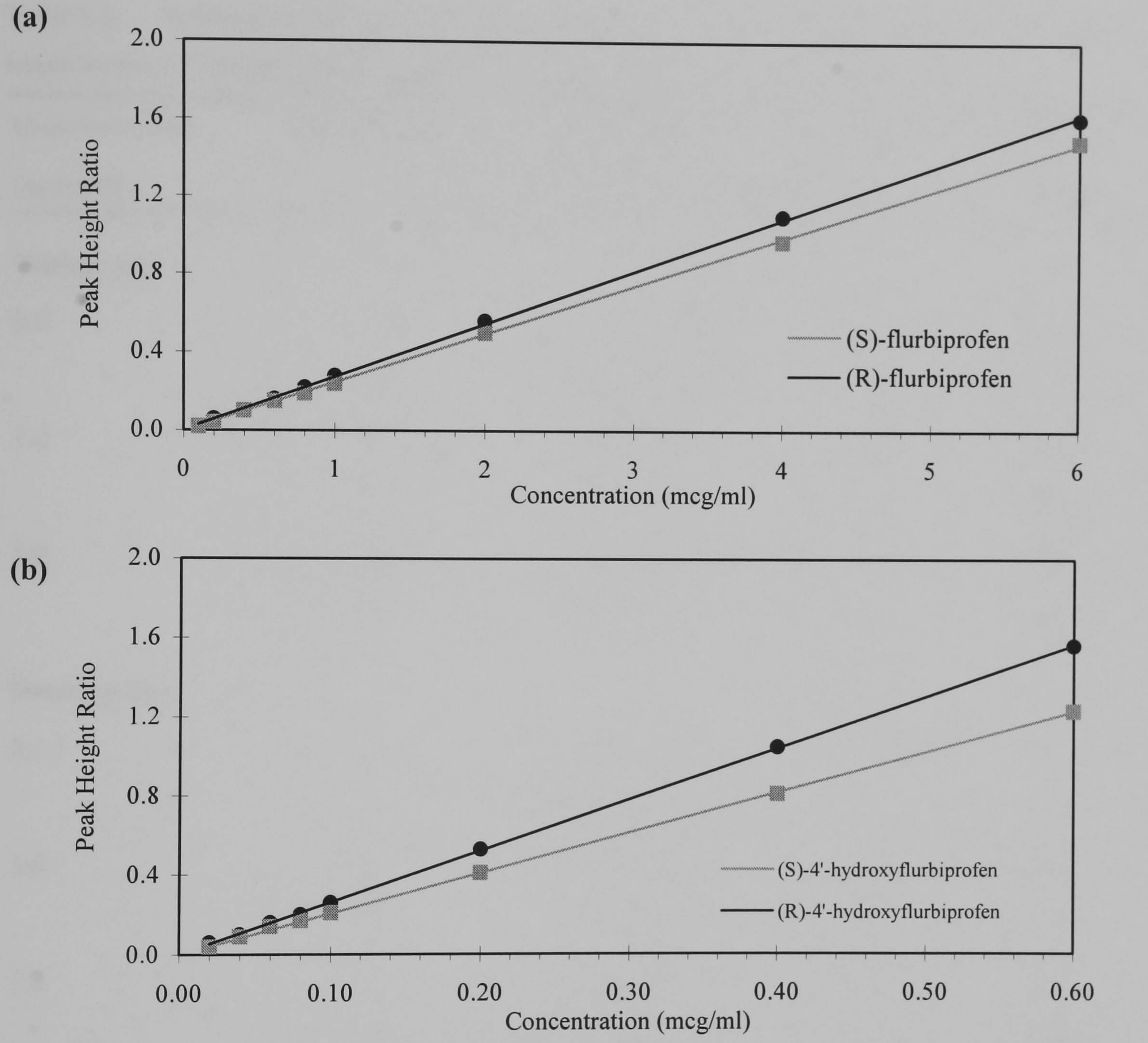


Figure 5.9: Typical calibration curves prepared for the quantification of the enantiomers of (a) flurbiprofen and (b) 4'-hydroxyflurbiprofen in serum samples using fluorescence detection.

Table 5.8: Within-day and between-day analytical variation and accuracy of flurbiprofen enantiomers in "spiked" serum samples (mean ± s.d.; n=6) *.

Concentration (mcg/ml)	Enantiomer	Concentration determined (mcg/ml)	C.V. (%)	M.D. (%)
Within-day				
0.2	*R*	0.199 ± 0.008	3.96	-0.5
	S	0.197 ± 0.008	3.85	-1.5
1.0	*R*	1.01 ± 0.03	3.04	1.0
	S	1.00 ± 0.03	3.41	0.0
5.0	*R*	5.04 ± 0.05	1.00	0.8
	S	5.04 ± 0.05	0.96	0.8
Between-day				
0.2	*R*	0.203 ± 0.011	5.36	1.5
	S	0.204 ± 0.008	3.77	2.0
1.0	*R*	1.02 ± 0.03	2.54	2.0
	S	1.01 ± 0.02	2.29	1.0
5.0	*R*	5.11 ± 0.07	1.46	2.2
	S	5.12 ± 0.08	1.63	2.4

* *C.V. = coefficient of variation; M.D. = mean difference.*

Table 5.9: Within-day and between-day analytical variation and accuracy of 4'-hydroxy-flurbiprofen enantiomers in "spiked" serum samples (mean ± s.d.; n=6) *.

Concentration (mcg/ml)	Enantiomer	Concentration determined (mcg/ml)	C.V. (%)	M.D. (%)
Within-day				
0.02	*R*	0.021 ± 0.001	2.67	5.0
	S	0.021 ± 0.001	1.96	5.0
0.1	*R*	0.099 ± 0.004	3.55	-1.0
	S	0.101 ± 0.005	4.82	1.0
0.5	*R*	0.51 ± 0.01	1.78	2.0
	S	0.50 ± 0.01	2.11	0.0
Between-day				
0.02	*R*	0.021 ± 0.001	4.72	5.0
	S	0.021 ± 0.001	4.26	5.0
0.1	*R*	0.099 ± 0.004	3.90	-1.0
	S	0.100 ± 0.005	5.26	0.0
0.5	*R*	0.51 ± 0.01	2.06	2.0
	S	0.51 ± 0.01	2.18	2.0

** C.V. = coefficient of variation; M.D. = mean difference.*

Table 5.10: Precision and accuracy data for the determination of a series of different enantiomeric compositions of flurbiprofen in serum at three different "total" concentrations (mean ± s.d.; n=3)*.

Enantiomer	Theoretical concentration(mcg/ml)	Measured concentration(mcg/ml)	C.V. (%)	M.D. (%)
Low concentration (0.5 mcg/ml)				
R	0.1	0.097 ± 0.002	1.79	-3.0
S	0.4	0.393 ± 0.005	1.28	-1.7
R	0.2	0.200 ± 0.007	3.26	0.0
S	0.3	0.295 ± 0.005	1.60	-1.6
R	0.3	0.316 ± 0.007	2.34	5.2
S	0.2	0.203 ± 0.005	2.33	1.3
R	0.4	0.409 ± 0.007	1.59	2.2
S	0.1	0.105 ± 0.007	6.78	4.7
Medium concentration (2.0 mcg/ml)				
R	0.4	0.402 ± 0.006	1.37	0.6
S	1.6	1.615 ± 0.019	1.15	1.0
R	0.8	0.811 ± 0.009	1.16	1.4
S	1.2	1.202 ± 0.028	2.37	0.2
R	1.2	1.193 ± 0.008	0.67	-0.6
S	0.8	0.813 ± 0.014	1.72	1.6
R	1.6	1.615 ± 0.009	0.56	1.0
S	0.4	0.408 ± 0.006	1.47	2.0
High concentration (6.0 mcg/ml)				
R	1.2	1.19 ± 0.007	0.60	-0.8
S	4.8	4.82 ± 0.024	0.50	0.3
R	2.4	2.40 ± 0.010	0.42	0.1
S	3.6	3.63 ± 0.031	0.84	0.8
R	3.6	3.61 ± 0.017	0.48	0.2
S	2.4	2.39 ± 0.010	0.40	-0.4
R	4.8	4.80 ± 0.009	0.19	0.0
S	1.2	1.19 ± 0.005	0.42	-0.7

* *C.V. = coefficient of variation; M.D. = mean difference.*

5.3.3 Preliminary *in vivo* investigations

Urinary excretion studies

The utility of the urine assay was demonstrated by analysing urine samples collected at 2 hour intervals from 0 to 12 hours followed by a pooled urine sample from 12 to 24 hours for three healthy volunteers following the oral administration of 100 mg of racemic flurbiprofen. Typical chromatograms of extracts obtained from non-hydrolysed and hydrolysed urine samples from this study are shown in Figure 5.10. The presence of 4'-hydroxyflurbiprofen at much higher concentrations than 3'-hydroxy-4'-methoxyflurbiprofen in urine and the necessity for a dual detection approach is clearly illustrated by the large differences observed in the size of the peaks between the enantiomers of the two metabolites.

The amounts of flurbiprofen and metabolites excreted in urine were determined in terms of free and both acyl- and phenol-conjugated levels for all the volunteers. Representative cumulative excretion profiles for a volunteer are shown in Figure 5.11 and the mean urinary excretion data is presented in Table 5.11. Of the administered dose some 19.3 % was excreted as flurbiprofen, 30.4 % as 4'-hydroxyflurbiprofen and 3.0 % as 3'-hydroxy-4'-methoxyflurbiprofen over the 24 hour collection period, primarily as conjugates. These results are in good agreement with previously reported results (Szpunar *et al.*, 1987; Knadler and Hall, 1989). It is noteworthy that the two metabolites have different preferential sites for conjugation; the 4'-hydroxy metabolite tends to form acyl-conjugates like the parent drug whereas the 3'-hydroxy-4'-methoxy metabolite is excreted mainly as phenol conjugates (see also Table 5.6). The formation of more stable phenol conjugates by 3'-hydroxy-4'-methoxyflurbiprofen maybe indicative of why it appeared to display slower kinetics during enzymatic hydrolysis.

A larger percentage of the dose was recovered in the (*R*)-form over the 24 hour collection period with total flurbiprofen and total 4'-hydroxyflurbiprofen having *S/R* ratios of 0.84 and 0.85 respectively, these findings are consistent with the observations of Knadler and Hall (1989). However, the minor metabolite, 3'-hydroxy-4'-methoxy-flurbiprofen, seemed to display preferential excretion of the (*S*)-isomer with a *S/R* ratio of 1.25. The proportion of metabolite present in recovered material seems to remain fairly constant with increasing urinary collection for both *R*- and *S*- forms, as can be seen in Figure 5.11d. In this case, the metabolite fractions for metabolites derived from

the (*R*)- and the (*S*)-enantiomers reached steady values of 0.62 and 0.65 respectively after 8 hours. The lack of variability in the metabolite fraction with increasing urinary collection was also observed with the two other volunteers.

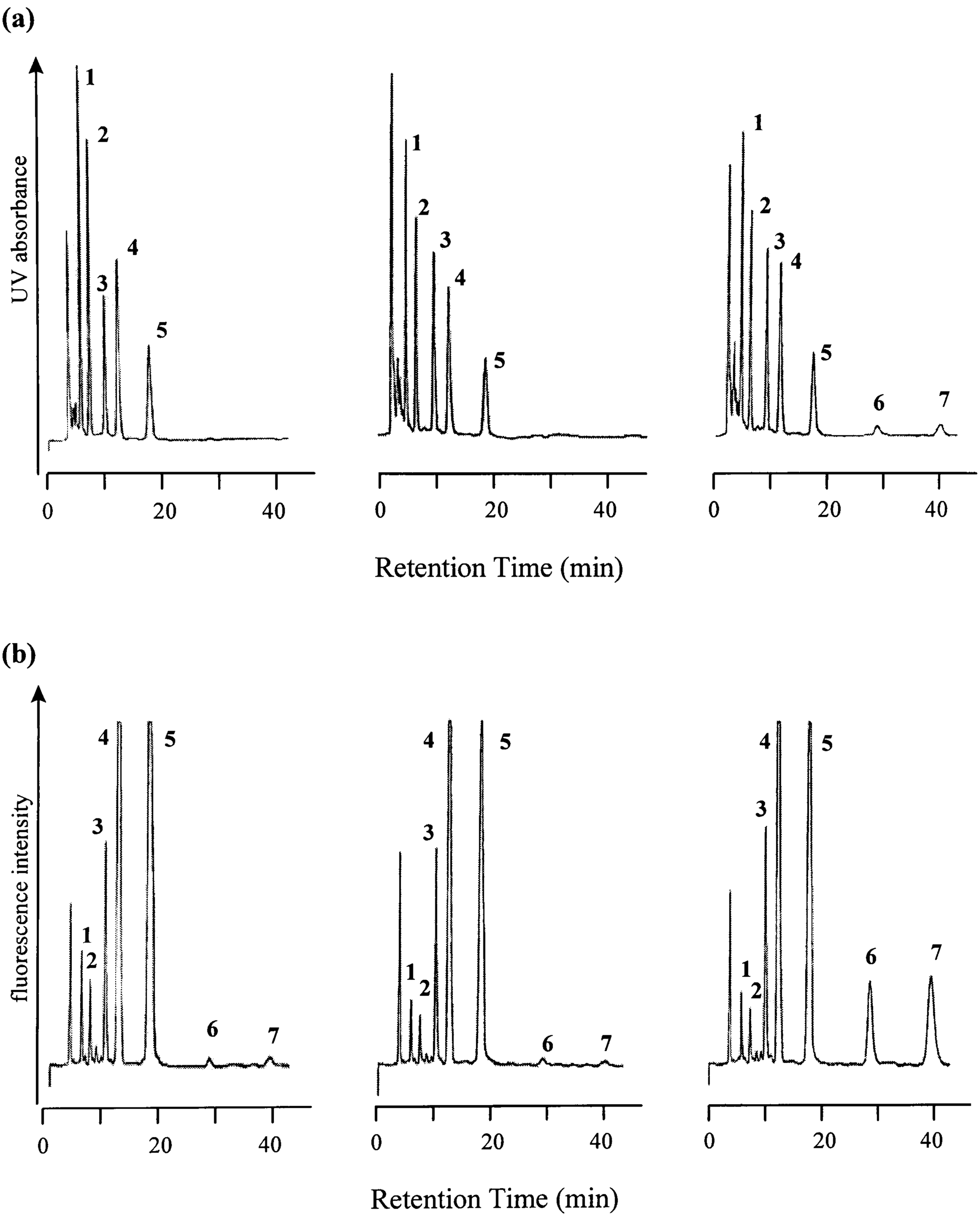


Figure 5.10: Chiral-phase chromatograms of (i) non-hydrolysed, (ii) base hydrolysed and (iii) enzymatic and base hydrolysed extracts of a urine sample from a volunteer obtained 4-6 hr following the oral administration of racemic flurbiprofen (100mg); using (a) UV detection and (b) fluorescence detection. Peaks : 1, (*R*)-flurbiprofen; 2, (*S*)-flurbiprofen; 3, (*S*)-naproxen (I.S.); 4, (*R*)-4'-hydroxyflurbiprofen; 5, (*S*)-4'-hydroxyflurbiprofen; 6, (*R*)-3'-hydroxy-4'-methoxyflurbiprofen and 7, (*S*)-3'-hydroxy-4'-methoxyflurbiprofen.

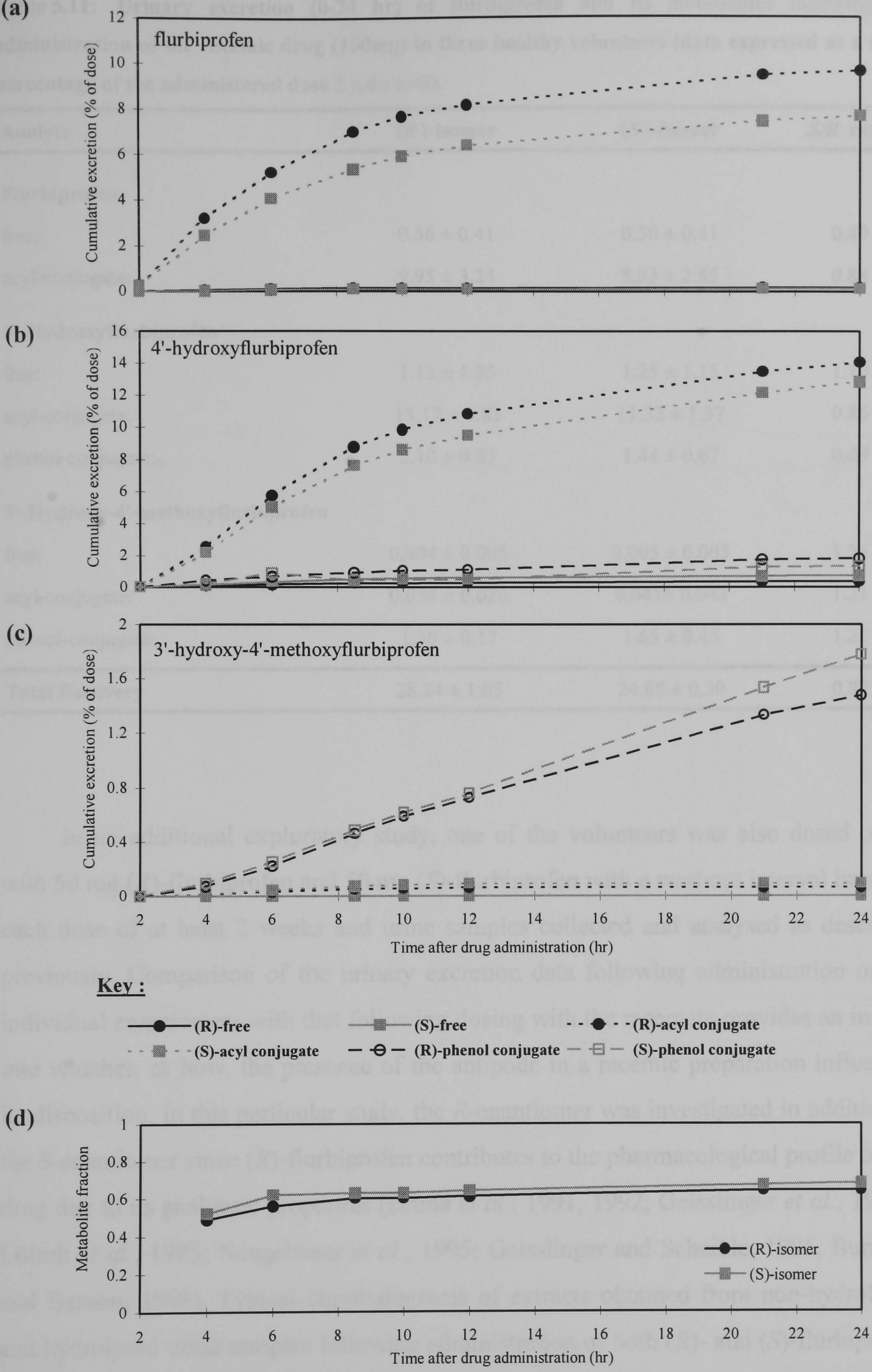


Figure 5.11: Cumulative urinary excretion profiles for (a) flurbiprofen, (b) 4'-hydroxyflurbiprofen and (c) 3'-hydroxy-4'-methoxyflurbiprofen following the oral administration of 100mg racemic flurbiprofen to a healthy volunteer and (d) corresponding metabolite fractions.

Table 5.11: Urinary excretion (0-24 hr) of flurbiprofen and its metabolites following the administration of the racemic drug (100mg) to three healthy volunteers (data expressed as a mean percentage of the administered dose ± s.d.; n=3).

Analyte	(*R*)-isomer	(*S*)-isomer	*S/R* ratio
Flurbiprofen			
free:	0.56 ± 0.41	0.50 ± 0.41	0.89
acyl-conjugate:	9.95 ± 3.24	8.33 ± 2.85	0.84
4'-Hydroxyflurbiprofen			
free:	1.13 ± 1.25	1.25 ± 1.15	1.10
acyl-conjugate:	13.17 ± 0.83	11.32 ± 1.37	0.86
phenol-conjugate:	2.10 ± 0.83	1.44 ± 0.67	0.69
3'-Hydroxy-4'-methoxyflurbiprofen			
free:	0.004 ± 0.005	0.005 ± 0.005	1.25
acyl-conjugate:	0.034 ± 0.026	0.041± 0.043	1.21
phenol-conjugate:	1.30 ± 0.17	1.63 ± 0.43	1.25
Total Recovery	28.24 ± 1.03	24.68 ± 0.30	0.87

In an additional exploratory study, one of the volunteers was also dosed orally with 50 mg (*R*)-flurbiprofen and 50 mg (*S*)-flurbiprofen with a washout interval between each dose of at least 2 weeks and urine samples collected and analysed as described previously. Comparison of the urinary excretion data following administration of the individual enantiomers with that following dosing with the racemate provides an insight into whether, or how, the presence of the antipode in a racemic preparation influences its disposition. In this particular study, the *R*-enantiomer was investigated in addition to the *S*-enantiomer since (*R*)-flurbiprofen contributes to the pharmacological profile of the drug due to its analgesic properties (Brune *et al.*, 1991, 1992; Geisslinger *et al.*, 1994a; Lötsch *et al.*, 1995; Neugebauer *et al.*, 1995; Geisslinger and Schaible, 1996, Buritova and Besson, 1998). Typical chromatograms of extracts obtained from non-hydrolysed and hydrolysed urine samples following administration of both (*R*)- and (*S*)-flurbiprofen are shown in Figures 5.12 and 5.13. The products detected in the urine samples retained the same stereochemical configuration as the administered enantiomer. This highlights the lack of chiral inversion of flurbiprofen in humans and is consistent with the findings of Jamali *et al.*, (1988) and Geisslinger *et al.* (1994b).

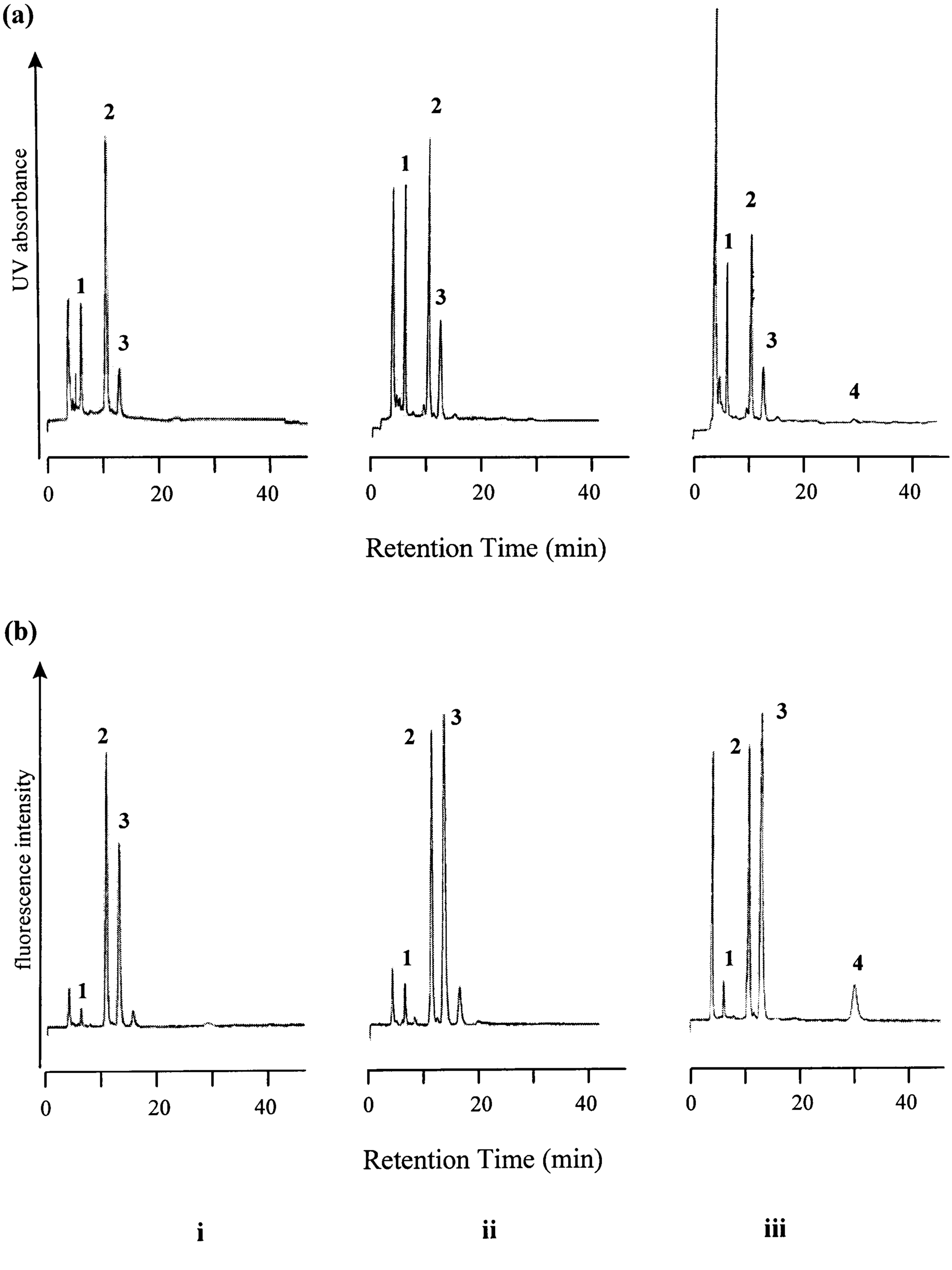


Figure 5.12: Chiral-phase chromatograms of (i) non-hydrolysed, (ii) base hydrolysed and (iii) enzymatic and base hydrolysed extracts of a urine sample from a volunteer obtained 4-6 hr following the oral administration of (*R*)-flurbiprofen (50mg); using (a) UV detection and (b) fluorescence detection. Peaks : 1, (*R*)-flurbiprofen; 2, (*S*)-naproxen (I.S.); 3, (*R*)-4'-hydroxy-flurbiprofen; and 4, (*R*)-3'-hydroxy-4'-methoxyflurbiprofen [Mobile phase hexane:ethanol (90:10 v/v) containing trifluoroacetic acid (0.05% v/v); Flow rate, 1.0ml/min].

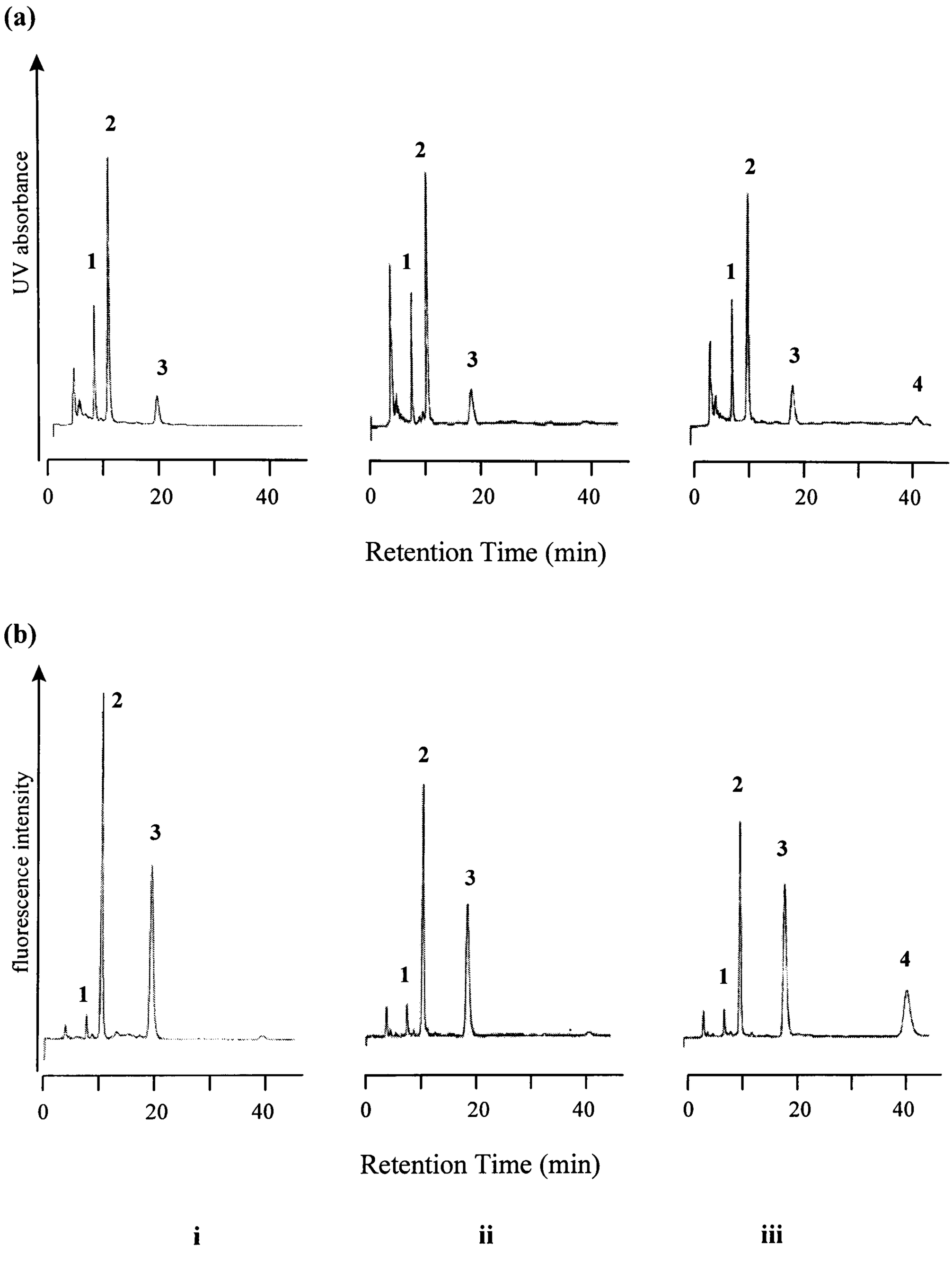


Figure 5.13: Chiral-phase chromatograms of (i) non-hydrolysed, (ii) base hydrolysed and (iii) enzymatic and base hydrolysed extracts of a urine sample from a volunteer obtained 4-6 hr following the oral administration of (*S*)-flurbiprofen (50mg); using (a) UV detection and (b) fluorescence detection. Peaks : 1, (*S*)-flurbiprofen; 2, (*S*)-naproxen (I.S.); 3, (*S*)-4'-hydroxy-flurbiprofen; and 4, (*S*)-3'-hydroxy-4'-methoxyflurbiprofen [Mobile phase hexane:ethanol (90:10 v/v) containing trifluoroacetic acid (0.05% v/v); Flow rate, 1.0ml/min].

The total recoveries of flurbiprofen and its metabolites following administration of the single enantiomers and the racemate to a healthy volunteer are presented in Table 5.12. It is worth noting that the recovery values following dosing with (*R,S*)-flurbiprofen are expressed as a percentage of the enantiomeric dose, rather than administered dose (as in Table 5.11), for ease of comparison. A greater proportion of the dose was recovered in 24 hours following administration of (*R*)-flurbiprofen than with (*S*)-flurbiprofen, this is in agreement with preferential elimination of products with the *R*-configuration following dosing with the racemate. The total recoveries of material in the *R*-isomeric form after administration of 50mg (*R*)-flurbiprofen and 100mg (*R,S*)-flurbiprofen were consistent and there was good correlation between the amounts of the individual products. Similar observations were also made for (*S*)-flurbiprofen with the only disparages being between the quantities of free (*S*)-flurbiprofen and acyl-conjugated (*S*)-4'-hydroxyflurbiprofen recovered. In general, this would suggests that there is little dispositional interaction between the enantiomers of flurbiprofen.

Table 5.12: Urinary excretion (0-24 hr) of flurbiprofen and its metabolites following the administration of (*R*)-flurbiprofen (50mg), (*R,S*)-flurbiprofen (100mg) and (*S*)-flurbiprofen (50mg) to a healthy volunteer (data expressed as a mean percentage of the enantiomeric dose) *.

Analyte	50mg	100mg racemate		50mg
	(*R*)-flurbiprofen	(*R*)-isomer	(*S*)-isomer	(*S*)-flurbiprofen
Flurbiprofen				
free:	0.52	0.36	0.26	2.61
acyl-conjugate:	19.94	19.34	15.36	15.89
4'-Hydroxyflurbiprofen				
free:	0.94	0.68	1.50	4.30
acyl-conjugate:	28.05	28.14	25.70	17.31
phenol-conjugate:	0.73	3.64	2.64	1.3
3'-Hydroxy-4'-methoxyflurbiprofen				
free:	N.D.	0.01	0.01	N.D.
acyl-conjugate:	N.D.	0.01	0.09	N.D.
phenol-conjugate:	2.47	2.96	3.56	4.82
Total Recovery	52.65	55.14	49.12	46.23

* *N.D. = not detected.*

Serum study

Following the administration of 100mg racemic flurbiprofen, blood samples as well as urine samples were collected from one of the volunteers. Serum was isolated and analysed using the methodology outlined in section 5.2.5 and typical chromatograms of a 3hr and a 8hr collection are shown in Figure 5.14. The additional peaks at 10.0 and 13.2 minutes were not observed in pre-dose serum or serum standard extracts and appear to become more prominent with time. These peaks could possibly correspond to the enantiomers of the 3',4'-dihydroxy- intermediate involved in the formation of 3'-hydroxy-4'-methoxyflurbiprofen. However, an authentic sample of 3',4'-dihydroxy-flurbiprofen was not readily available for confirmation and any further investigations of the suspected metabolite were not thought to be essential as it was considered to be outside the scope of the current studies.

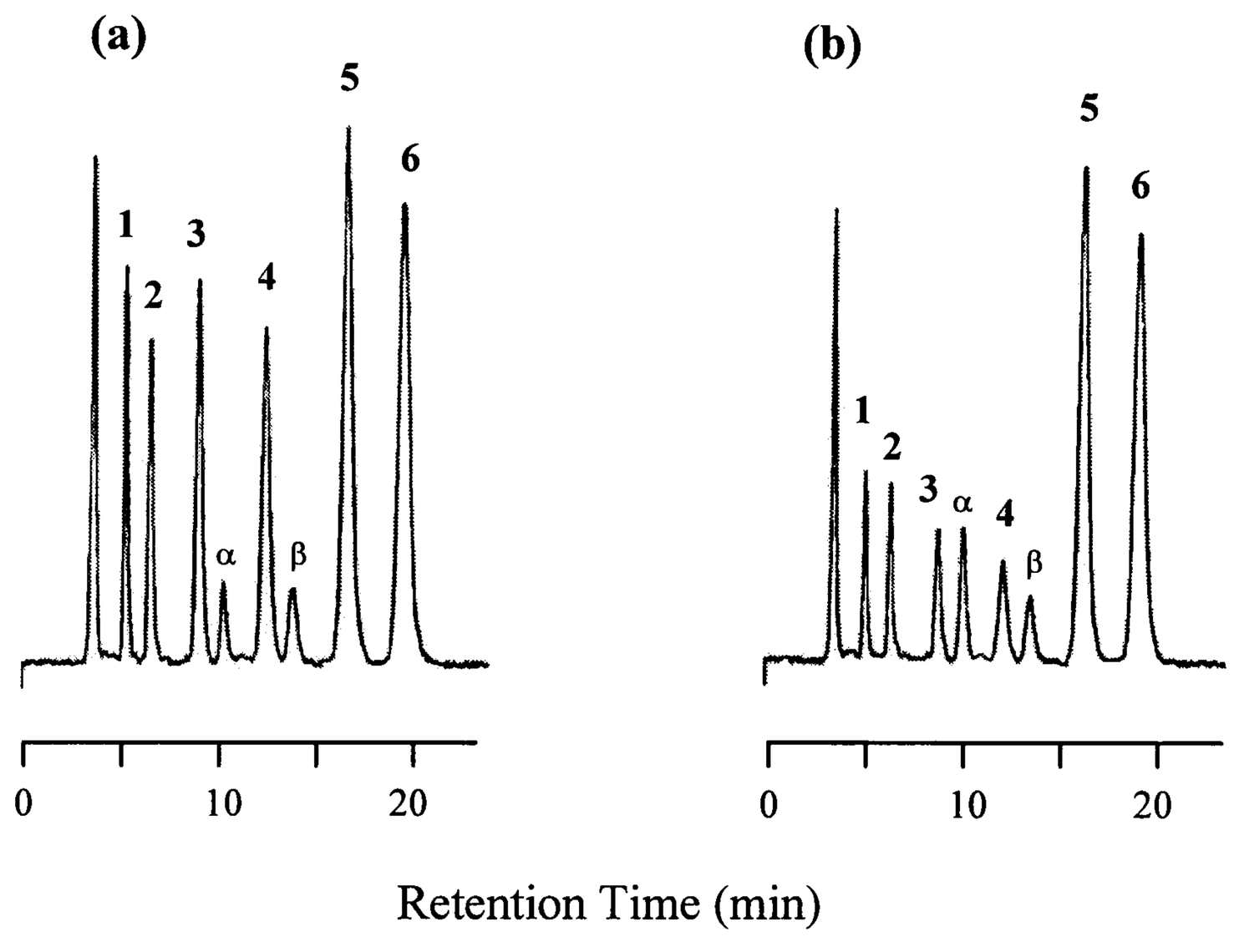


Figure 5.14: Chiral-phase chromatograms of serum samples (a) 3 hr and (b) 8 hr following oral administration of (*R,S*)-flurbiprofen (100mg) to a healthy volunteer. Peaks : 1, (*R*)-flurbiprofen; 2, (*S*)-flurbiprofen; 3, (*R*)-4'-hydroxy-flurbiprofen; 4, (*S*)-4'-hydroxyflurbiprofen; 5, (*R*)-benoxa-profen (I.S.) and 6, (*S*)-benoxaprofen; α and β, additional unknown peaks [Mobile phase hexane:ethanol (87:13 v/v) containing TFA (0.05% v/v); Flow rate, 1.0ml/min].

Semilogarithmic plots of serum concentration-time profiles of flurbiprofen and 4'-hydroxyflurbiprofen for the volunteer are shown in Figure 5.15. Visual examination of the profiles for (*R*)- and (*S*)-flurbiprofen suggested that the drug exhibited non-stereoselective pharmacokinetics and that disappearance of the drug from systemic circulation was biphasic. The enantiomers of the 4'-hydroxy metabolite were present in serum at concentrations between ten to twenty times less than simultaneous flurbiprofen concentrations and their rates of disappearance from serum appeared to be comparable to that of the parent enantiomers.

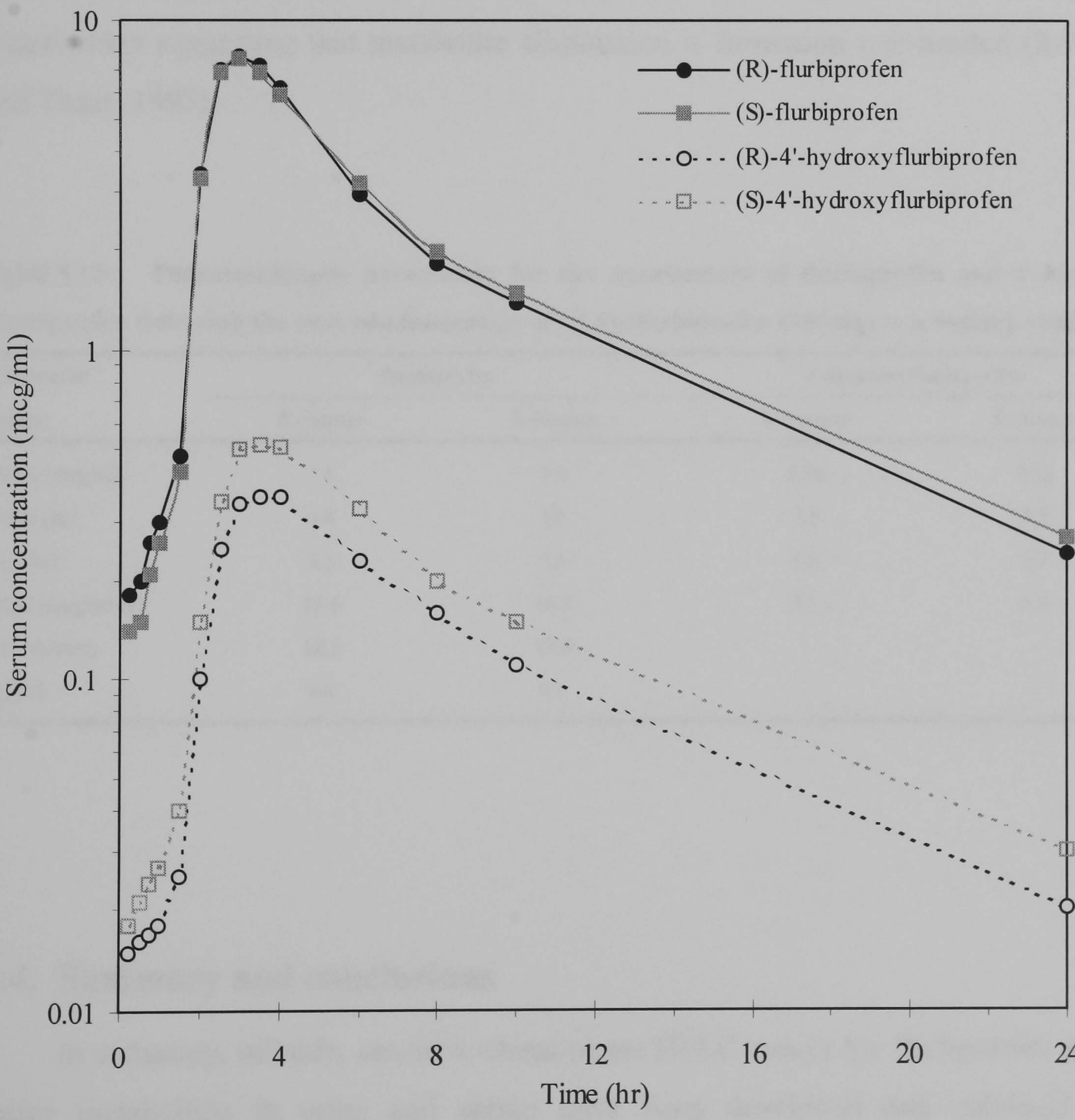


Figure 5.15: Serum concentration-time profiles for the enantiomers of flurbiprofen and 4'-hydroxyflurbiprofen following the oral administration of (*R,S*)-flurbiprofen (100mg) to a healthy volunteer.

Pharmacokinetic data analysis of the serum concentrations were performed using the procedures described in Chapter 6 and the results are presented in Table 5.13. There were only small differences in the values of the parameters between (*R*)- and (*S*)-flurbiprofen and between (*R*)- and (*S*)-4'-hydroxyflurbiprofen, which reiterates that neither the drug or metabolite appear to undergo extensive stereoselective disposition in this individual. The observation that (*R*)- and (*S*)-flurbiprofen have equivalent terminal half-lives is consistent with the findings of Jamali *et al.* (1988). The AUC for the (*S*)-4'-hydroxy metabolite was 9.5 % of the AUC of (*S*)-flurbiprofen and the corresponding value for the antipode was 6.9 %, emphasising that the serum concentrations of the enantiomers of this metabolite are relatively low. The terminal half-lives for (*R*)- and (*S*)- 4'-hydroxy-flurbiprofen were essentially equivalent to those of the parent enantiomers suggesting that metabolite elimination is formation rate-limited (Rowland and Tozer, 1995).

Table 5.13: Pharmacokinetic parameters for the enantiomers of flurbiprofen and 4'-hydroxy-flurbiprofen following the oral administration of (*R*,*S*)-flurbiprofen (100mg) to a healthy volunteer.

Parameter	**flurbiprofen**		**4'-hydroxyflurbiprofen**	
(units)	***R*-isomer**	***S*-isomer**	***R*-isomer**	***S*-isomer**
Cmax (mcg/ml)	7.8	7.6	0.36	0.52
Tmax (hr)	3.0	3.0	3.5	3.5
$t_{1/2,z}$ (hr)	5.5	5.6	5.8	5.9
AUC (mcg/ml hr)	45.0	46.3	3.1	4.4
CL (ml/min)	18.5	18.0	-	-
V_d (L)	8.8	8.8	-	-

5.4. Summary and conclusions

In summary, reliable, sensitive chiral-phase HPLC assays for flurbiprofen and its major metabolites in urine and serum have been developed and validated. The compounds being extracted from urine or serum using liquid-liquid extraction techniques prior to separation and resolution on an amylose tris (3,5-dimethylphenyl carbamate) CSP (Chiralpak AD). The use of a fluorescence detector and a UV detector in series allows for the concurrent determination of the enantiomers of flurbiprofen,

4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen in urine extracts. The employment of a fluorescence detector is sufficient for the quantitative analysis of the enantiomers of flurbiprofen and the 4'-hydroxy metabolite in serum.

The methods were applied in preliminary urinary excretion and serum disposition studies following the administration of flurbiprofen to healthy volunteers. The urinary recovery studies indicate that the acyl-conjugates of flurbiprofen and the 4'-hydroxy metabolite are the major components in urine the excretion profile of the drug shows modest enantioselectivity for products with the *R*-configuration. The analysis of serum samples from a volunteer suggest enantioselectivity in the disposition of flurbiprofen in humans is limited.

To our knowledge this is the first report of methodology allowing simultaneous determination of the enantiomers of flurbiprofen and its major metabolites in biological fluids using chiral-phase chromatography.

CHAPTER 6 :

Stereoselective disposition of flurbiprofen in young and elderly volunteers

6.1. Introduction

Numerous studies have investigated the plasma pharmacokinetics of the enantiomers of flurbiprofen following either administration of the racemate or the individual enantiomers (Jamali *et al.*, 1988, 1991; Small *et al.*, 1990; Cefali *et al.*, 1991; Young *et al.*, 1991; Knadler *et al.*, 1992a, 1992b; Geisslinger *et al.*, 1994b). Plasma concentrations of (*S*)-flurbiprofen were consistently higher than those of (*R*)-flurbiprofen in all these studies such that the area under the plasma concentration versus time curve of the *S*-enantiomer were significantly greater than that of the *R*-enantiomer. Statistically significant differences between flurbiprofen enantiomers were also found for systemic clearance (Jamali *et al.*, 1988; Small *et al.*, 1990; Young *et al.*, 1991; Knadler *et al.*, 1992a, 1992b) and half-life (Jamali *et al.*, 1991; Knadler *et al.*, 1992a; Geisslinger *et al.*, 1994b), indicating the faster elimination of the *R*-enantiomer. In contrast with the above findings, a recent human serum study suggests that flurbiprofen does not exhibit enantioselective pharmacokinetics (Oelkers *et al.*, 1997).

In comparison to ibuprofen, flurbiprofen demonstrates modest enantioselectivity in its disposition in humans. This is not surprising, since the disposition of flurbiprofen in man, unlike other species, is not complicated by the significant unidirectional *R*- to *S*-inversion observed with ibuprofen (Menzel-Soglowek *et al.*, 1992; Geisslinger *et al.*, 1994b). Flurbiprofen does not form a coenzyme A thioester *in vitro* (Knihinicki *et al.*, 1989; Knadler and Hall, 1990), a prerequisite for chiral inversion, and individual enantiomer administration to humans does not result in formation of its antipode (Jamali *et al.*, 1988; Geisslinger *et al.*, 1994b; Oelkers *et al.*, 1997). Furthermore, the negligible mutual enantiomeric interactions displayed by flurbiprofen at doses commonly employed may contribute to the lack of complexity in its disposition (Knadler *et al.*, 1989).

Differences in the disposition of flurbiprofen enantiomers are likely to arise due to enantioselectivity in plasma protein binding and elimination. The principle pathways of flurbiprofen elimination are through the formation of oxidation products and conjugates and therefore the stereoselectivity of these different processes need to be addressed. However, as a consequence of the difficulties associated with developing suitable methods for the enantiospecific bioanalysis of flurbiprofen with its metabolites, few human studies have considered this aspect of flurbiprofen disposition. The excretion of flurbiprofen as the acyl glucuronide accounts for about 20 % of the dose, and *in vitro*

experiments, using human hepatic microsomal preparations, reveal the glucuronidation to be stereoselective with (*R*)-flurbiprofen being a better substrate than (*S*)-flurbiprofen (Hamdoune *et al.*, 1995). Quantitatively, the major metabolic pathway for flurbiprofen is via oxidation to yield 4'-hydroxyflurbiprofen. Small *et al.* (1990) using an unpublished analytical method, suggested there was a lack of stereoselectivity in the disposition of this product in humans following administration of the drug. Urinary excretion studies indicate a predominance of products with the *R*-configuration in the recovered material, as greater amounts of the *R*-enantiomer were found both as total flurbiprofen (conjugated and unconjugated) and 4'-hydroxyflurbiprofen (Knadler *et al.*, 1992a, 1992b).

Studies on the influence of age on the pharmacokinetics and disposition of flurbiprofen in humans are scarce. In the study of Hamdy *et al.* (1980), the pharmacokinetics of flurbiprofen were evaluated after a single 50 mg dose of the racemate in 12 male osteoarthritis-patients (age range: 66-90 years, mean: 79 years) and 12 female osteoarthritis-patients (age range: 74-94 years, mean: 85 years) and compared the data obtained with parameters determined from previous studies in undefined healthy younger volunteers (age range: 18-40 years). Limited pharmacokinetic parameters were calculated, e.g. peak serum concentration and area under the plasma concentration versus time curve, based on an undocumented analytical procedure and no differences in the disposition of "total" flurbiprofen between young and elderly subjects were observed. A more extensive study performed by Kean *et al.*(1992) investigated the pharmacokinetics of flurbiprofen in 13 elderly (age range: 65-83 years, mean: 73 years) and 12 younger (age range: 40-60 years, mean: 53 years) patients with rheumatoid arthritis at the beginning, mid-point and end of an eight day, 100 mg twice daily, multiple-dose regimen. Renal clearance was decreased significantly in the elderly; however, renal clearance averaged only 10 % of total body clearance in these patients and is only a minor pathway of drug elimination compared with metabolism. The above investigations would suggest that age has minimal influence on the handling of flurbiprofen in humans, but such conclusions may be misleading as the lack of stereochemical analysis may mask age-associated differences in enantiomeric disposition.

The objective of the current investigation is to examine the disposition of flurbiprofen enantiomers and the formation of known metabolites following the oral administration of the racemic drug to healthy young and elderly volunteers.

Enantiospecific analysis will be performed using the analytical methodologies based on chiral phase HPLC described and validated in the previous Chapter.

6.2. Experimental

6.2.1 Clinical study protocol

Volunteers

Two groups of subjects were studied : a) four young healthy volunteers aged from 24 to 29 years (mean ± s.d., 26.8 ± 2.2 years), two male and two female; and b) four elderly healthy volunteers aged from 72 to 87 years (78.5 ± 7.2 years), two male and two female; their demographic data is shown in Appendix 10.Volunteers were judged to be in good health on the basis of case history, physical examination, electrocardiogram, and routine laboratory data including blood pressure and pulse. The study was approved by the Research Ethics Committee of the School of Medicine and Dentistry, King's College London. Volunteers were fully informed about the investigational procedure and the use of flurbiprofen. In compliance with the Declaration of Helsinki, written informed consent was obtained from each participant before enrolment in the study.

Study design

The volunteers were required to abstain from any alcohol, caffeine-based products and medication for at least 24 hours prior to the study. The volunteers were fasted for at least 8 hours overnight before a single 100mg oral dose of racemic flurbiprofen (Froben® tablet) was administered with 150ml of water. Food and drink were withheld for at least 3 hours after drug administration.

Blood samples (10 ml) were collected immediately before drug administration and at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 10, 12 and 24 hours post dosing in plain silicone coated vacutainer tubes. The venous blood samples were drawn for the initial 12 hour period via an indwelling cannula, to avoid repeated venipuncture, placed in a convenient vein in the arm of the volunteer. The cannula was flushed after each sample collection with 0.9% NaCl solution B.P. to avoid clotting in the cannula. Samples were

allowed to clot for two hours and serum obtained after centrifugation (10 min at 2000g). Urine samples were collected every two hours for the first 12 hours and then at 22 and 24 hours post drug administration. The individual urine volumes were recorded and a pooled 24 hour sample was prepared from the individual collections. Serum and urine samples were frozen at -20°C until required for analysis.

6.2.2 Chromatographic analysis

The enantiomeric composition of flurbiprofen and 4'-hydroxyflurbiprofen in serum and flurbiprofen, 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen, both free and conjugated, in urine were determined using the methodology described in Chapter 5. All samples were analysed in duplicate and a set of appropriate calibration standards analysed on each day.

6.2.3 Pharmacokinetic and data analysis

Determination of pharmacokinetic parameters was performed by conventional non-compartmental analysis of the enantiomeric serum concentration-time profiles of flurbiprofen and the 4'-hydroxy metabolite (Rowland and Tozer, 1995).

Maximum serum concentrations (C_{max}) and the corresponding times (T_{max}) were determined by examination of the individual serum concentration-time curves for each volunteer. The terminal elimination half-life ($t_{1/2,z}$) was determined using :

$$\mathbf{t_{1/2,z} = \ln 2/\lambda_z} \qquad \text{(Eqn. 6.1)}$$

where the elimination rate constant (λ_z) is obtained by log-linear regression of the terminal phase of the serum concentration-time profile. The area under the curve (AUC) was calculated by :

$$\mathbf{AUC = AUC_{0\rightarrow t} + AUC_{t\rightarrow \infty}} \qquad \text{(Eqn. 6.2)}$$

where AUC up to the last measurable data point ($AUC_{0\rightarrow t}$) was determined using the trapezoidal rule and extrapolated to infinite time ($AUC_{t\rightarrow\infty}$) by dividing the last measurable serum concentration by λ_z. The average extrapolated area accounted for 8%

and 16% of the total area for the enantiomers of flurbiprofen and 4'-hydroxyflurbiprofen respectively. Apparent total body clearance (CL) and volume of distribution (V_d) of flurbiprofen were calculated from the following expressions:

$$\mathbf{CL = F.Dose/AUC} \qquad \text{(Eqn. 6.3)}$$

$$\mathbf{V_d = F.Dose/\lambda_z.AUC} \qquad \text{(Eqn. 6.4)}$$

where the fraction of the dose reaching systemic circulation (F) was assumed to be unity. This is reasonable, since previous studies have shown that greater than 95 % of orally administered 50 mg doses of the racemate were excreted in urine within 48 hours post dosing (Risdall *et al.*, 1978) and the bioavailability of different tablet dosages relative to an oral solution averaged 94 % (Spzunar *et al.*, 1987).

The fraction of each flurbiprofen enantiomer converted to oxidation products (F_{ox}), namely 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen, was calculated from an examination of the total recoveries (free and conjugated) of the individual enantiomers of flurbiprofen and metabolites in urine over 24 hours using:

$$\mathbf{F_{ox} = \frac{\%\ oxidative\ metabolites}{\%\ total\ recovery}} \qquad \text{(Eqn. 6.5)}$$

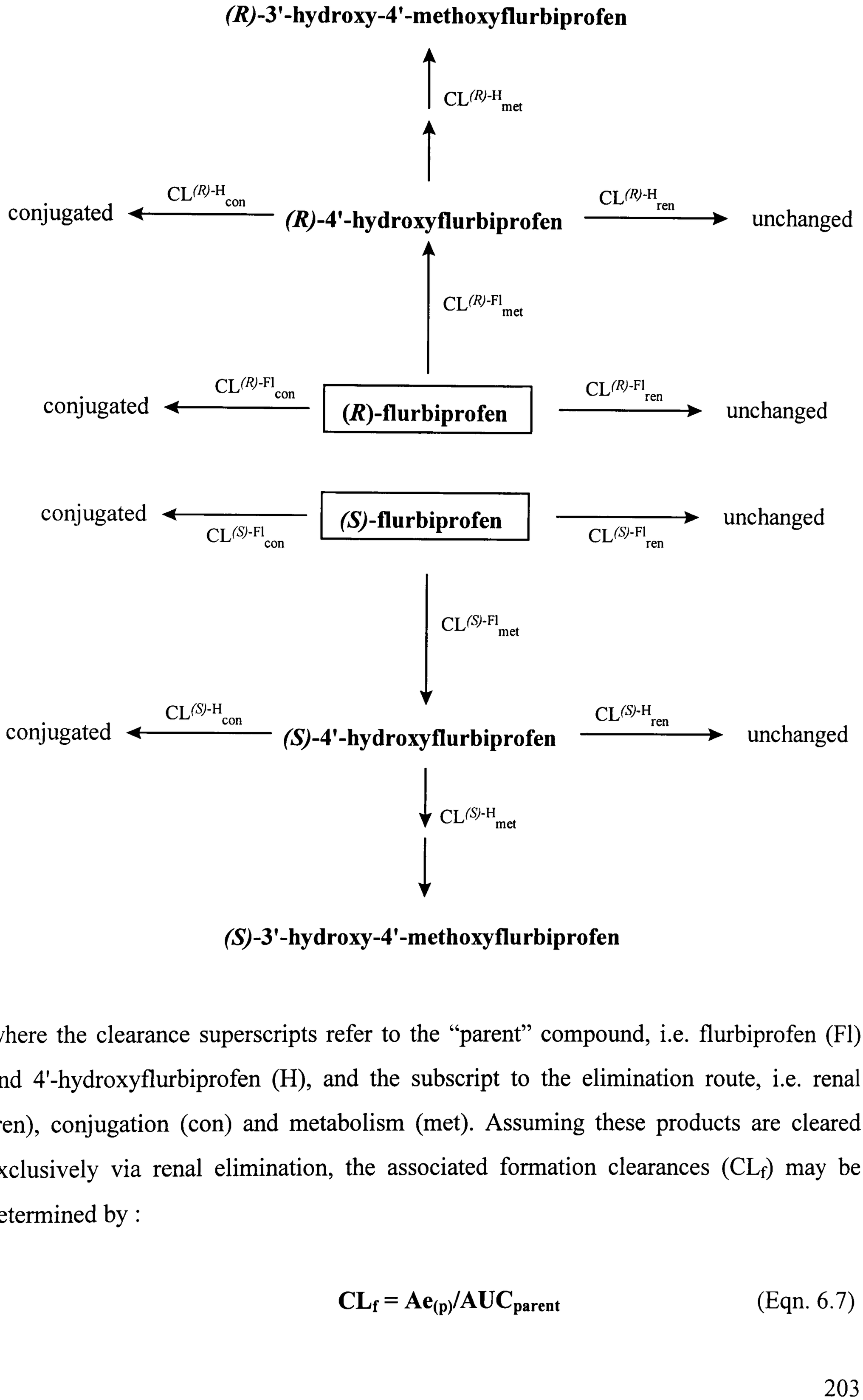
where "% oxidative metabolites" is the sum of the molar amounts of the 4'-hydroxy and 3'-hydroxy-4'-methoxy metabolites excreted, both free plus conjugated, expressed as a percentage of the administered dose and "% total recovery" is the percentage of the dose recovered in urine.

The clearance of 4'-hydroxyflurbiprofen (CL_m) was estimated from :

$$\mathbf{CL_m = F_{ox}.(F.Dose)/AUC_m} \qquad \text{(Eqn. 6.6)}$$

where AUC_m is the area under the 4'-hydroxyflurbiprofen serum concentration-time curve corrected for molar equivalency to the parent drug.

When administered to humans, the major dispositional pathways of the enantiomers of flurbiprofen can be considered in terms of the following scheme :



where the clearance superscripts refer to the "parent" compound, i.e. flurbiprofen (Fl) and 4'-hydroxyflurbiprofen (H), and the subscript to the elimination route, i.e. renal (ren), conjugation (con) and metabolism (met). Assuming these products are cleared exclusively via renal elimination, the associated formation clearances (CL_f) may be determined by :

$$\mathbf{CL_f = Ae_{(p)}/AUC_{parent}} \qquad \text{(Eqn. 6.7)}$$

where $Ae_{(p)}$ is the total amount of product recovered in urine either unchanged or conjugated, and in the case of the 4'-hydroxy product includes the amount undergoing further oxidation to the 3'-hydroxy-4'-methoxy metabolite; and AUC_{parent} is the AUC of the compound from which the product was derived (Rowland and Tozer, 1995).

The mean pharmacokinetic parameters derived from the serum data and urinary excretion data for the enantiomers of flurbiprofen, 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen were tested for stereoselective differences using the Student's t-test for paired samples in both age groups. Age-associated differences in the pharmacokinetic data and urinary data of both (*R*)- and (*S*)-flurbiprofen were assessed using the Student's t-test for independent samples.

6.3. Results

6.3.1 Serum kinetics

Enantioselective differences

The mean serum concentration-time profiles for the enantiomers of flurbiprofen and 4'-hydroxyflurbiprofen following the administration of 100 mg of the racemate to four healthy young volunteers are shown in Figure 6.1, and the individual serum level data are tabulated in Appendix 11. The pharmacokinetic parameters derived from the serum concentrations of the enantiomers of flurbiprofen and the magnitude of the enantiomeric differences for these parameters, expressed as *S/R* ratios, are presented in Tables 6.1 and 6.2 respectively. Likewise, the individual enantiomer pharmacokinetic parameters and corresponding *S/R* ratios for 4'-hydroxyflurbiprofen are shown in Tables 6.3 and 6.4 respectively.

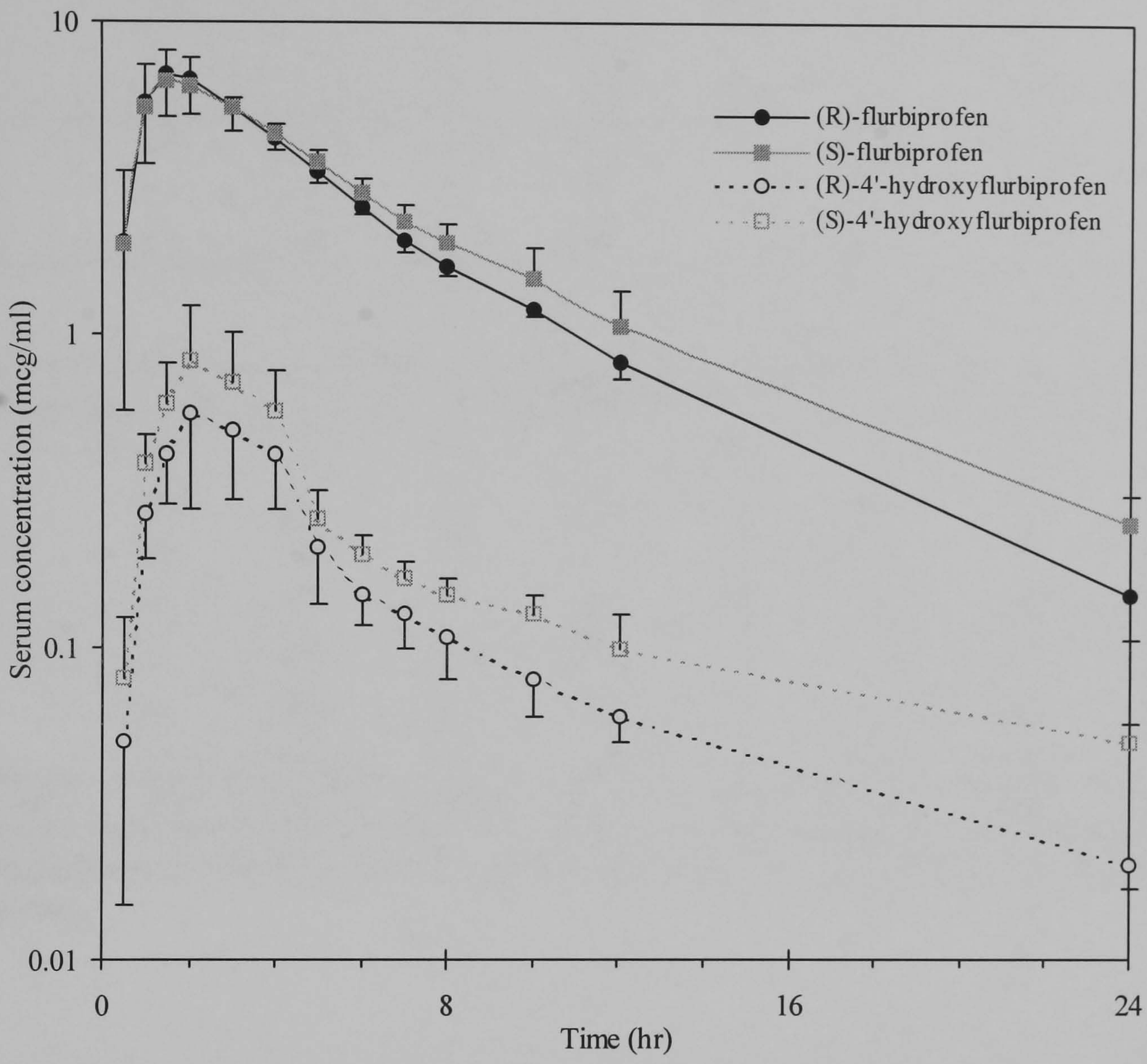


Figure 6.1: Mean serum concentration-time profiles for the enantiomers of flurbiprofen and 4'-hydroxyflurbiprofen following the oral administration of (*R,S*)-flurbiprofen (100 mg) to four healthy young volunteers (mean ± s.d.).

Table 6.1: Pharmacokinetic parameters of flurbiprofen enantiomers following the oral administration of the racemic drug (100 mg) to four healthy young volunteers.

				(*R*)-flurbiprofen				
Subject Code	**C_{max} (mcg/ml)**	**T_{max} (hr)**	**$t_{1/2,z}$ (hr)**	**AUC (mcg/ml hr)**	**CL (ml/min)**	**CL_{ren} (ml/min)**	**Vd (L)**	**F_{ox}**
Fy1	6.3	2.0	4.0	43.1	19.4	1.96	6.8	0.69
Fy2	8.9	1.5	4.2	45.7	18.3	0.54	6.6	0.77
Fy3	7.7	1.5	4.6	41.0	20.3	0.94	8.0	0.70
Fy4	4.6	1.5	5.3	39.6	21.0	1.35	9.7	0.66
Mean	6.9	1.6	4.5	42.3	19.7	1.20	7.8	0.71
s.d.	1.8	0.3	0.6	2.6	1.2	0.61	1.4	0.05
C.V. %	26.4	15.4	12.6	6.2	6.1	50.6	18.2	6.6
				(*S*)-flurbiprofen				
Fy1	5.5	3.0	4.7	43.4	19.2	1.85	7.8	0.66
Fy2	8.3	1.5	4.8	54.9	15.2	0.39	6.4	0.77
Fy3	7.5	2.0	5.1	46.7	17.8	0.66	7.8	0.73
Fy4	4.8	3.0	6.3	52.9	15.8	0.85	8.5	0.68
Mean	6.5	2.4	5.2	49.5	17.0	0.94	7.6	0.71
s.d.	1.6	0.8	0.7	5.3	1.9	0.64	0.9	0.05
C.V. %	25.2	31.6	13.7	10.8	10.9	67.9	12.0	7.0
p (*R* vs. *S*) *	N.S.	N.S.	p<0.005	N.S.	N.S.	N.S.	N.S.	N.S.

** Comparison between the means for the enantiomers of flurbiprofen were carried out using Student's t-test for paired samples (N.S. = p>0.05).*

Table 6.2: Pharmacokinetic parameter ratios (*S/R*) of flurbiprofen following the oral administration of the racemic drug (100 mg) to four healthy young volunteers.

				ratio (*S/R*)				
Subject Code	**C_{max}**	**T_{max}**	**$t_{1/2,z}$**	**AUC**	**CL**	**CL_{ren}**	**Vd**	**F_{ox}**
Fy1	0.87	1.50	1.16	1.01	0.99	0.94	1.15	0.96
Fy2	0.94	1.00	1.15	1.20	0.83	0.72	0.96	1.00
Fy3	0.97	1.33	1.11	1.14	0.88	0.70	0.98	1.04
Fy4	1.04	2.00	1.17	1.33	0.75	0.63	0.88	1.03
Mean	0.96	1.46	1.15	1.17	0.86	0.75	0.99	1.01
s.d.	0.07	0.42	0.03	0.14	0.10	0.14	0.11	0.04
C.V. %	7.44	28.57	2.33	11.55	11.70	18.09	11.33	3.81

Table 6.3: Pharmacokinetic parameters of 4'-hydroxyflurbiprofen enantiomers following the oral administration of (*R*,*S*)-flurbiprofen (100 mg) to four healthy young volunteers.

			(*R*)-4'-hydroxyflurbiprofen			
Subject Code	**C_{max} (mcg/ml)**	**T_{max} (hr)**	**$t_{1/2,z}$ (hr)**	**AUC (mcg/ml hr)**	**CL (ml/min)**	**CL_{ren} (ml/min)**
Fy1	0.43	3.0	6.1	3.11	195.9	53.7
Fy2	0.93	2.0	5.6	4.39	155.6	15.3
Fy3	0.59	2.0	7.8	3.14	199.3	26.1
Fy4	0.28	2.0	7.4	2.34	251.1	42.8
Mean	0.56	2.3	6.7	3.25	200.5	34.5
s.d.	0.28	0.5	1.1	0.85	39.1	17.1
C.V. %	50.0	22.2	15.8	26.1	19.5	49.6
			(*S*)-4'-hydroxyflurbiprofen			
Fy1	0.59	2.0	12.8	5.77	101.6	26.1
Fy2	1.33	2.0	7.4	6.04	112.8	9.9
Fy3	0.95	2.0	10.1	5.68	113.6	14.1
Fy4	0.39	2.0	12.1	3.97	150.9	23.6
Mean	0.82	2.0	10.6	5.37	119.7	18.4
s.d.	0.41	0.0	2.4	0.94	21.5	7.7
C.V. %	50.8	0.0	23.1	17.6	18.0	41.8
p (*R* vs. *S*) *	p<0.05	N.S.	p<0.05	p<0.005	p<0.01	N.S.

** Comparison between the means for the enantiomers of 4'-hydroxyflurbiprofen were carried out using Student's t-test for paired samples (N.S. = p>0.05).*

Table 6.4: Pharmacokinetic parameter ratios (*S/R*) of 4'-hydroxyflurbiprofen following the oral administration of (*R*,*S*)-flurbiprofen (100 mg) to four healthy young volunteers.

			ratio (*S/R*)			
Subject Code	**C_{max}**	**T_{max}**	**$t_{1/2,z}$**	**AUC**	**CL**	**CL_{ren}**
Fy1	1.37	0.67	2.10	1.86	0.52	0.49
Fy2	1.43	1.00	1.32	1.38	0.72	0.65
Fy3	1.61	1.00	1.29	1.81	0.57	0.54
Fy4	1.39	1.00	1.62	1.70	0.60	0.55
Mean	1.45	0.92	1.58	1.68	0.60	0.56
s.d.	0.11	0.17	0.38	0.22	0.09	0.07
C.V. %	7.5	18.2	23.8	12.8	14.5	12.2

Following administration of 100 mg of the racemate, the serum levels of (*R*)-flurbiprofen were greater than those of the *S*-enantiomer during the initial 3 hour post-dose period, i.e. mainly during the absorption phase; beyond this point, the serum concentrations of (*S*)-flurbiprofen were consistently higher those of its antipode. However, the differences between the serum concentrations of the enantiomers did not reach statistical significance at any sampling time during the 24 hour period. The (*S*)-flurbiprofen serum levels declined more slowly and this is reflected by the significantly longer half-life ($t_{1/2,z}$) values ($p<0.005$; Table 6.1). These findings are consistent with previously reported data (Jamali *et al.*, 1991; Knadler *et al.*, 1992a; Geisslinger *et al.*, 1994b). None of the other pharmacokinetic parameters were significantly different between the individual enantiomers and the lack of enantioselectivity in the pharmacokinetics of flurbiprofen in the young volunteers is highlighted by the modest deviations of *S/R* parameter ratios from unity (Tables 6.1 and 6.2). It is of interest to note that both enantiomers of flurbiprofen were cleared primarily by metabolism with renal clearance accounting for less than 6 % of the total clearance (Table 6.1).

The metabolite, 4'-hydroxyflurbiprofen, was also detectable in serum samples, the sensitivity of the analytical method was such that the concentrations of the metabolite enantiomers could be determined in the 24 hr sample. It is worth noting that the assay method previously employed by Knadler *et al.* (1992a) lacked sensitivity capable of quantifying the levels of both (*R*)- and (*S*)-4'-hydroxyflurbiprofen beyond 13 hours post dosing and consequently only limited pharmacokinetic parameters for this metabolite have been reported previously.

The mean serum concentration-time curves for (*R*)- and (*S*)-4'-hydroxyflurbiprofen in comparison with those of (*R*)- and (*S*)-flurbiprofen for the young volunteers are shown in Figure 6.1. The enantiomers of the 4'-hydroxy metabolite were typically present in serum at concentrations between five to twenty fold less than those of the corresponding flurbiprofen concentrations and their elimination displayed a more prominent biphasic characteristic with the terminal half lives being larger than those of the parent enantiomers. In contrast to flurbiprofen, the serum levels of the *S*-enantiomer of 4'-hydroxyflurbiprofen always exceeded those of its antipode with the differences being statistically significant at all sampling times with the exception of those at 4 and 5 hours. As a consequence, the area under the curve (AUC) and maximum serum concentration (C_{max}) of (*S*)-4'-hydroxyflurbiprofen were significantly greater ($p<0.05$) than for (*R*)-4'-hydroxyflurbiprofen (Table 6.3). The terminal half-life ($t_{1/2,z}$) and total

clearance (CL) also showed statistically significant differences, with (*S*)-4'-hydroxy-flurbiprofen having a longer $t_{1/2,z}$ and a smaller CL.

The mean serum concentration-time profiles for the enantiomers of flurbiprofen and 4'-hydroxyflurbiprofen following administration of the racemic drug to four healthy elderly volunteers are shown in Figure 6.2, and the individual serum level data are tabulated in Appendix 12. The associated pharmacokinetic parameters and *S/R* ratios derived for flurbiprofen are presented in Tables 6.5 and 6.6 respectively; and likewise for 4'-hydroxyflurbiprofen in Tables 6.7 and 6.8.

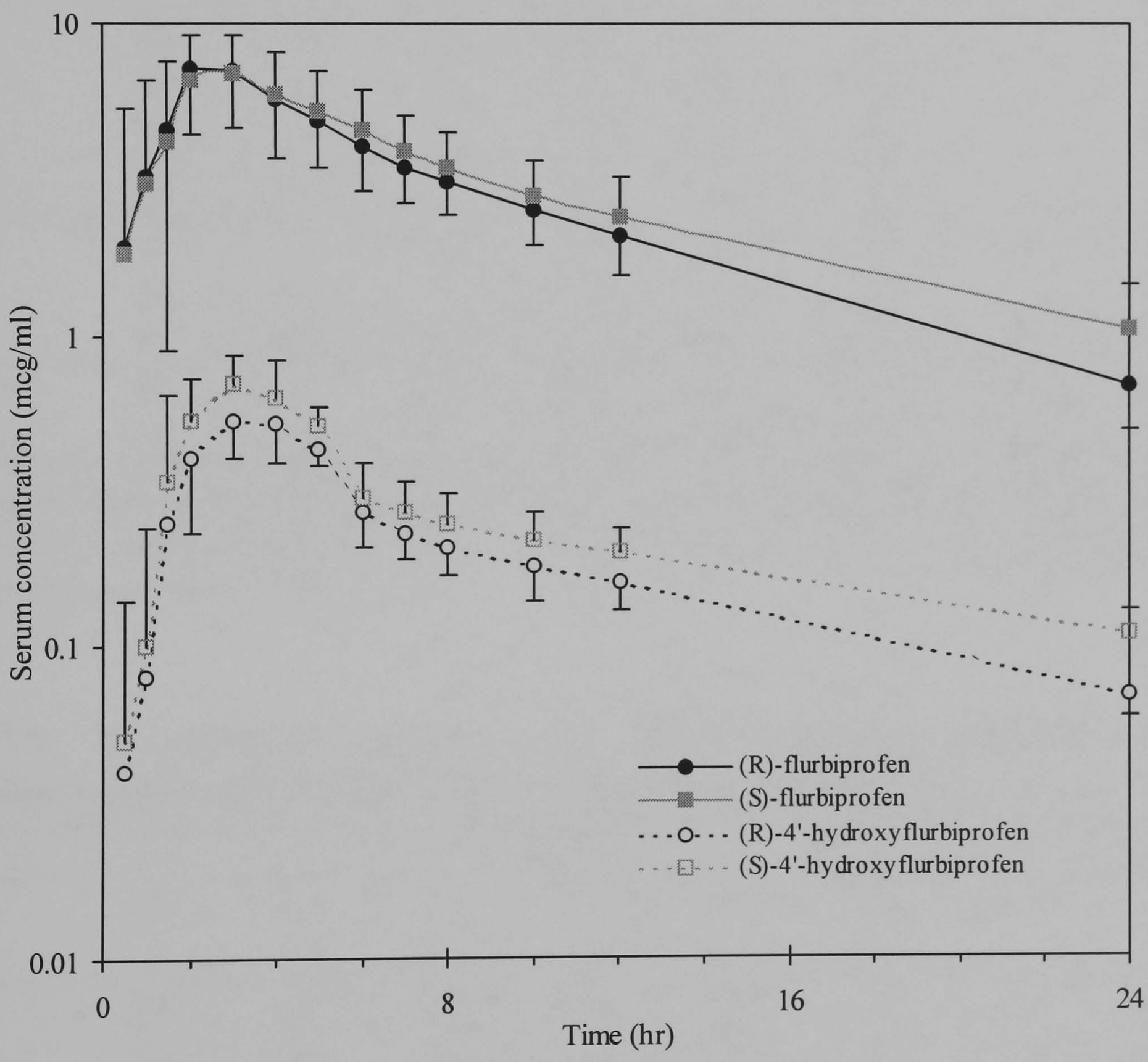


Figure 6.2: Mean serum concentration-time profiles for the enantiomers of flurbiprofen and 4'-hydroxyflurbiprofen following the oral administration of (*R,S*)-flurbiprofen (100 mg) to four healthy elderly volunteers (mean ± s.d.).

Table 6.5: Pharmacokinetic parameters of flurbiprofen enantiomers following the oral administration of the racemic drug (100 mg) to four healthy elderly volunteers.

				(*R*)-flurbiprofen				
Subject Code	**C_{max} (mcg/ml)**	**T_{max} (hr)**	**$t_{1/2,z}$ (hr)**	**AUC (mcg/ml hr)**	**CL (ml/min)**	**CL_{ren} (ml/min)**	**Vd (L)**	**F_{ox}**
Fe1	10.9	3.0	7.2	83.5	10.0	2.07	6.2	0.55
Fe2	5.6	3.0	7.7	57.0	14.6	1.00	9.7	0.71
Fe3	8.1	1.0	8.0	74.5	11.2	0.21	7.7	0.68
Fe4	8.9	2.0	5.7	63.0	13.2	2.49	6.6	0.66
Mean	8.4	2.3	7.1	69.5	12.3	1.44	7.6	0.65
s.d.	2.2	1.0	1.0	11.8	2.1	1.03	1.6	0.07
C.V. %	26.5	42.6	13.9	17.0	16.9	71.7	21.1	10.7
p(Y vs. E) †	N.S.	N.S.	p<0.01	p<0.05	p<0.005	N.S.	N.S.	N.S.
				(*S*)-flurbiprofen				
Fe1	10.2	3.0	9.7	98.0	8.5	1.39	7.1	0.57
Fe2	5.1	3.0	10.2	64.2	13.0	0.69	11.5	0.74
Fe3	7.7	1.0	10.6	84.2	9.9	0.15	9.1	0.66
Fe4	8.2	2.0	7.9	70.9	11.8	1.96	8.1	0.63
Mean	7.8	2.3	9.6	79.3	10.8	1.05	8.9	0.65
s.d.	2.1	1.0	1.2	15.0	2.0	0.79	1.9	0.07
C.V. %	26.7	42.6	12.4	18.9	18.4	75.6	21.1	10.9
p(R vs. S) *	N.S.	N.S.	p<0.001	p<0.01	p<0.005	N.S.	p<0.05	N.S.
p(Y vs. E) †	N.S.	N.S.	p<0.005	p<0.05	p<0.01	N.S.	N.S.	N.S.

** Comparison between the means for the enantiomers of flurbiprofen were carried out using Student's t-test for paired samples (N.S. = p>0.05).* † *Comparison between respective means for young and elderly volunteers were carried out using Student's t-test for independent samples (N.S. = p>0.05).*

Table 6.6: Pharmacokinetic parameter ratio (*S/R*) of flurbiprofen following the oral administration of the racemic drug (100 mg) to four healthy elderly volunteers.

				ratio (*S/R*)				
Subject Code	**C_{max}**	**T_{max}**	**$t_{1/2,z}$**	**AUC**	**CL**	**CL_{ren}**	**Vd**	**F_{ox}**
Fe1	0.93	1.00	1.35	1.17	0.85	0.67	1.15	1.04
Fe2	0.92	1.00	1.33	1.13	0.89	0.69	1.18	1.04
Fe3	0.95	1.00	1.33	1.13	0.88	0.71	1.18	0.97
Fe4	0.92	1.00	1.38	1.13	0.89	0.79	1.23	0.95
Mean	0.93	1.00	1.35	1.14	0.88	0.72	1.18	1.00
s.d.	0.01	0.00	0.02	0.02	0.02	0.05	0.03	0.04
C.V. %	1.48	0.00	1.70	2.06	2.02	7.09	2.68	4.48
p(Y vs. E) †	N.S.	N.S.	p < 0.001	N.S.	N.S.	N.S.	p < 0.05	N.S.

† *Comparison between respective means for young and elderly volunteers were carried out using Student's t-test for independent samples (N.S. = p>0.05).*

Table 6.7: Pharmacokinetic parameters of 4'-hydroxyflurbiprofen enantiomers following the oral administration of (*R,S*)-flurbiprofen (100 mg) to four healthy elderly volunteers.

			(*R*)-4'-hydroxyflurbiprofen			
Subject Code	**C_{max} (mcg/ml)**	**T_{max} (hr)**	**$t_{1/2,z}$ (hr)**	**AUC (mcg/ml hr)**	**CL (ml/min)**	**CL_{ren} (ml/min)**
Fe1	0.71	4.0	9.7	5.88	83.5	34.3
Fe2	0.48	5.0	12.0	5.54	113.8	22.8
Fe3	0.63	3.0	9.5	6.74	89.9	5.1
Fe4	0.49	3.0	9.5	4.51	129.2	65.0
Mean	0.58	3.8	10.2	5.67	104.1	31.8
s.d.	0.11	1.0	1.2	0.92	21.2	25.2
C.V. %	19.4	25.5	11.7	16.3	20.4	79.2
p(Y vs. E) †	N.S.	p<0.05	p<0.01	p<0.01	p<0.01	N.S.
			(*S*)-4'-hydroxyflurbiprofen			
Fe1	0.93	4.0	13.1	9.97	50.3	17.4
Fe2	0.61	5.0	15.6	7.63	86.6	15.2
Fe3	0.77	2.0	11.7	7.92	73.7	3.4
Fe4	0.62	3.0	14.4	6.79	82.5	35.2
Mean	0.73	3.5	13.7	8.08	73.3	17.8
s.d.	0.15	1.3	1.7	1.35	16.2	13.1
C.V. %	20.6	36.9	12.4	16.7	22.1	73.8
p(R vs. S) *	p<0.01	N.S.	p<0.01	p<0.05	p<0.05	N.S.
p(Y vs. E) †	N.S.	N.S.	N.S.	p<0.05	P<0.05	N.S.

** Comparison between the means for the enantiomers of 4'-hydroxyflurbiprofen were carried out using Student's t-test for paired samples (N.S. = p>0.05).* † *Comparison between respective means for young and elderly volunteers were carried out using Student's t-test for independent samples (N.S. = p>0.05).*

Table 6.8: Pharmacokinetic parameter ratios (*S/R*) of 4'-hydroxyflurbiprofen following the oral administration of (*R,S*)-flurbiprofen (100 mg) to four healthy elderly volunteers.

			ratio (*S/R*)			
Subject Code	**C_{max}**	**T_{max}**	**$t_{1/2,z}$**	**AUC**	**CL**	**CL_{ren}**
Fe1	1.31	1.00	1.35	1.70	0.60	0.51
Fe2	1.27	1.00	1.30	1.38	0.76	0.66
Fe3	1.22	0.67	1.22	1.18	0.82	0.68
Fe4	1.27	1.00	1.51	1.51	0.64	0.54
Mean	1.27	0.92	1.35	1.44	0.71	0.60
s.d.	0.04	0.17	0.12	0.22	0.10	0.09
C.V. %	2.8	18.2	9.0	15.2	14.5	14.3
p(Y vs. E) †	p<0.05	N.S.	N.S.	N.S.	N.S.	N.S.

† *Comparison between respective means for young and elderly volunteers were carried out using Student's t-test for independent samples (N.S. = p>0.05).*

Following the oral administration of 100mg racemic flurbiprofen, the relative serum concentration time profiles for (*R*)- and (*S*)-flurbiprofen followed a similar trend to that observed in the young group, however in this case the gradual enrichment of (*S*)-flurbiprofen in serum during the elimination phase resulted in statistical significant differences in the serum levels between the two enantiomers beyond 8 hours post dosing (Figure 6.2). As would be expected, the half live of (*S*)-flurbiprofen was also significantly longer than that of its antipode in this age group (Table 6.5). However, it appears that greater enantioselectivity is observed in the disposition of flurbiprofen in the elderly compared to the young since other statistically significant differences were observed between the individual enantiomers with (*S*)-flurbiprofen displaying a larger AUC, reduced CL, and a greater volume of distribution (V_d) than (*R*)-flurbiprofen (Table 6.5). An interesting observation in this age group is the notable consistency of the *S/R* ratio between individuals for a given pharmacokinetic parameter, the highest inter-subject variability observed being only 7 % (Table 6.6).

The serum concentration time profiles of (*R*)- and (*S*)- 4'-hydroxyflurbiprofen, following the administration of the drug to the elderly volunteers, mirrored the observations in the young group. The metabolite being present at concentrations up to 20 fold less than the parent and the consistent predominance of the *S*-enantiomer in serum (Figure 6.2). This is reflected in the pharmacokinetic parameters determined for the 4'-hydroxy metabolite displaying the same enantioselective differences as those previously observed in the young, with the *S*-enantiomer having a higher C_{max}, longer $t_{1/2,z}$, reduced CL and a larger AUC in comparison to (*R*)-4'-hydroxyflurbiprofen (Table 6.7).

Age-associated differences

Comparison between the young and elderly groups indicated age-related differences with respect to the pharmacokinetics of (*R*)- and (*S*)-flurbiprofen (Table 6.5). The elderly volunteers exhibited significantly longer $t_{1/2,z}$ and reduced CL for both enantiomers of the drug. The accumulation of flurbiprofen in the elderly is reflected in the group having significantly larger AUC values for (*R*)- and (*S*)-flurbiprofen in comparison to the corresponding values observed for the young volunteers. (Tables 6.1 and 6.5). Age-associated differences were also observed in the pharmacokinetic parameters determined for 4'-hydroxyflurbiprofen with both enantiomers of the metabolite displaying significantly larger AUCs and a reduction in clearance in the

elderly volunteers (Tables 6.3 and 6.7). Additional differences between the two age groups were seen in the case of the *R*-enantiomer, i.e. the elderly exhibited significantly greater values of T_{max} and $t_{1/2,z}$ for (*R*)-4'-hydroxyflurbiprofen.

These age-related changes in the pharmacokinetics of the enantiomers of flurbiprofen and 4'-hydroxyflurbiprofen are associated with only modest alterations in enantioselectivity. This is evident from the majority of the parameter *S/R* ratios displaying good agreement between the two age groups (Tables 6.6 and 6.8). The observation of an enantioselective difference in the volume of distribution of flurbiprofen in the elderly (*R*: 7.6 vs. *S*: 8.9 L) which was not evident in the young (*R*: 7.8 vs. *S*: 7.6 L) is reflected in a significant difference in the *S/R* ratio for this parameter between the two groups (Tables 6.2 and 6.6). The significantly greater *S/R* ratio seen for $t_{1/2,z}$ of flurbiprofen in the elderly is probably as a result of this age-related appearance of enantioselectivity in the V_d, as age had no influence on the enantioselective clearance of the drug (Table 6.2 and 6.6). In addition a significant, but modest, change in the *S/R* ratio for C_{max} of 4'-hydroxyflurbiprofen was apparent, such that the elderly showed a reduced enantioselective difference (Table 6.4 and 6.8).

6.3.2 Urinary excretion

Enantioselective differences

The urinary excretion data and formation clearance values for flurbiprofen and its metabolites for the young volunteer group are summarised in Tables 6.9 and 6.10 respectively (the individual data are tabulated in Appendices 13 to 15). The total amount of flurbiprofen and metabolites recovered in the 0-24 hour collection period was 72.3 ± 4.6 % and these values compare favourably with previously published values of 65 to 79 % (Szpunar *et al.*, 1987; Cefali *et al.*, 1991; Knadler *et al.*, 1992a). The principle pathways of flurbiprofen elimination involve the formation of flurbiprofen glucuronide and 4'-hydroxyflurbiprofen with recovery values of 15.7 % and 46.8 % respectively. The 3'-hydroxy-4'-methoxy metabolite is a minor component accounting for only 4.2 % of the administered dose excreted in urine (Table 6.9). In general, a larger percentage of the dose was recovered with products having the *R*-configuration (*S/R* = 0.87), which is in agreement with the findings of Knadler *et al.*(1992a, 1992b).

The excretion of flurbiprofen as the acyl glucuronide demonstrated a preference for the *R*-enantiomer conjugate (*S/R* = 0.86; Table 6.9). This pattern is reflected in all four volunteers displaying higher formation clearances for (*R*)-flurbiprofen compared to (*S*)-flurbiprofen glucuronidation, but the difference did not reach statistical significance (p = 0.07; Table 6.10). It is worth noting that (*R*)-flurbiprofen was also predominant in the amount of free drug excreted (*S/R* = 0.89), but the difference in renal clearance between the enantiomers did not achieve statistical significant (p = 0.06; Table 6.1).

Enantioselective clearance of flurbiprofen via oxidation to 4'-hydroxyflurbiprofen is also evident from the formation clearances, which were significantly greater for the metabolite derived from the *R*-enantiomer compared to its *S*-antipode (Table 6.10). The excretion of 4'-hydroxyflurbiprofen is primarily as conjugates and the formation of these metabolites displays regioselectivity and enantioselectivity. That is, conjugation of the 4'-hydroxy metabolite favours the generation of acyl glucuronides rather than phenolic conjugates (ratio acyl/phenol: *S* = 14.9; *R* = 14.0) and (*R*)-4'-hydroxyflurbiprofen acyl-glucuronide rather than (*S*)-4'-hydroxyflurbiprofen acyl-glucuronide (*S/R* = 0.83; Table 6.9). The stereoselective clearance of (*R*)-4'-hydroxyflurbiprofen via conjugation is also indicated in the formation clearances, with the *R*-enantiomer being cleared significantly faster than the antipode (Table 6.10). As with the parent drug, a modest higher urinary recovery (*S/R* = 0.92), paralleled with a higher renal clearance, is observed for the *R*-enantiomer of the nonconjugated metabolite (Tables 6.3 and 6.9). However, relatively high inter-subject variability in the values of these parameters for both enantiomers resulted in the differences not achieving statistical significance.

Clearance of 4'-hydroxyflurbiprofen through the formation of the 3'-hydroxy-4'-methoxy metabolite is a minor elimination pathway and appears to lack substrate enantioselectivity, however higher formation clearances for the *R*-enantiomer were observed in three of the four volunteers (Table 6.10). It is noteworthy, that conjugation of this metabolite may display opposing regioselectivity and stereoselectivity to that of the 4'-hydroxy metabolite, favouring the formation of phenolic conjugates of the *S*-enantiomer (Table 6.9).

Table 6.9: Urinary excretion (0-24 hr) of flurbiprofen and its metabolites following the oral administration of the racemic drug (100 mg) to four healthy young volunteers (data expressed as a mean percentage of the administered dose).

Analyte	*R*-enantiomer mean ± s.d.	*S*-enantiomer mean ± s.d.	*S/R* ratio
Flurbiprofen			
free:	2.99 ± 1.55	2.67 ± 1.55 *	0.89
acyl-conjugate:	8.43 ± 1.51	7.26 ± 1.03 *	0.86
total	11.42 ± 2.34	9.92 ± 2.19 *	0.87
4'-Hydroxyflurbiprofen			
free:	5.86 ± 2.48	5.40 ± 2.19	0.92
acyl-conjugate:	18.18 ± 1.84	15.05 ± 1.60 *	0.83
phenol-conjugate:	1.30 ± 1.34	1.01 ± 1.18	0.78
total	25.33 ± 1.45	21.46 ± 0.65 *	0.85
3'-Hydroxy-4'-methoxyflurbiprofen			
free:	0.021 ± 0.030	0.024 ± 0.004	1.14
acyl-conjugate:	0.029 ± 0.034	0.022 ± 0.027	0.76
phenol-conjugate:	1.87 ± 0.27	2.20 ± 0.33	1.18
total	1.92 ± 0.30	2.24 ± 0.37	1.17
Total Recovery	38.67 ± 2.63	33.63 ± 2.03 *	0.87

** $p < 0.05$ for comparison between the means of enantiomers using Student's t-test for paired samples.*

Table 6.10: Metabolite formation clearances (ml/min) following the oral administration of racemic flurbiprofen (100 mg) to four healthy young volunteers.

(a) Flurbiprofen

Subject	acyl glucuronide			4'-hydroxyflurbiprofen		
Code	*R*	*S*	*S/R* ratio	*R*	*S*	*S/R* ratio
Fy1	3.00	2.82	0.94	10.88	9.04	0.83
Fy2	2.56	1.89	0.74	10.36	7.50	0.72
Fy3	3.43	2.43	0.71	10.28	8.19	0.80
Fy4	4.43	2.72	0.61	11.43	7.45	0.65
Mean	3.36	2.47	0.75	10.74	8.05	0.75
s.d.	0.80	0.42	0.14	0.53	0.74	0.08
C.V. %	23.84	16.94	18.29	4.96	9.25	10.61
p (*R* vs. *S*) *	N.S.			p<0.05		

(b) 4'-Hydroxyflurbiprofen

Subject	conjugate(s)			3'-hydroxy-4'-methoxyflurbiprofen		
Code	*R*	*S*	*S/R* ratio	*R*	*S*	*S/R* ratio
Fy1	93.9	39.1	0.42	13.07	7.39	0.57
Fy2	92.6	55.1	0.60	6.99	7.63	1.09
Fy3	105.5	50.6	0.48	11.41	7.00	0.61
Fy4	151.1	74.3	0.49	12.33	7.79	0.63
Mean	110.8	54.8	0.50	10.95	7.45	0.73
s.d.	27.5	14.6	0.07	2.73	0.34	0.25
C.V. %	24.83	26.72	14.93	24.89	4.61	33.85
p(R vs. S) *	p<0.01			N.S.		

** Comparison between the means for the enantiomers were carried out using Student's t-test for paired samples (N.S. = p>0.05).*

The urinary excretion data and formation clearance values for flurbiprofen and its metabolites for the elderly volunteer group are summarised in Tables 6.11 and 6.12 respectively (the individual data are tabulated in Appendices 16 to 18). The total amount of flurbiprofen and metabolites recovered in the 0-24 hour collection period was 60.6 ± 10.7 %. The 4'-hydroxy and 3'-hydroxy-4'-methoxy metabolites accounting for 36.0 and 2.9 % of the administered dose excreted in urine respectively. Flurbiprofen, was excreted in approximately equal amounts as the free drug (11.0 %) and the acyl glucuronide (10.6 %;) accounting for 21.6 % of the administered dose (Table 6.11).

The excretion of flurbiprofen in the elderly volunteers displayed similar enantioselectivity to that observed in the young volunteer group, with significantly greater amounts of free drug and acyl glucuronide recovered in urine having the *R*-configuration (free *S/R* = 0.83, acyl glucuronide *S/R* = 0.79; Table 6.11). This pattern of excretion is reflected in (*R*)-flurbiprofen displaying higher values for renal and glucuronide formation clearances than its antipode in all four volunteers (Table 6.5 and 6.12).

Examination of the formation clearances for 4'-hydroxyflurbiprofen arising from oxidation of either enantiomer of the drug indicates substrate selectivity favouring (*R*)-flurbiprofen (*S/R* = 0.71; Table 6.12). Renal excretion of the metabolite showed the same preference for the *R*-enantiomer as observed with the parent compound (Table 6.11). As with the young, the elimination of the 4'-hydroxy metabolite via conjugation favours the formation of ester-glucuronides and the *R*-enantiomer (Tables 6.11 and 6.12). The clearance of 4'-hydroxyflurbiprofen via further metabolism to 3'-hydroxy-4'-methoxyflurbiprofen showed a distinct enantioselectivity in favour of the *R*-enantiomer (Table 6.12). However, in contrast to the young group the elimination of the 3'-hydroxy-4'-methoxy metabolite as phenolic conjugates appeared to have a preference for the *R*- rather than the *S*-enantiomer (Table 6.11).

Table 6.11: Urinary excretion (0-24 hr) of flurbiprofen and its metabolites following the oral administration of the racemic drug (100 mg) to four healthy elderly volunteers (data expressed as a mean percentage of the administered dose).

Analyte	*R*-enantiomer mean ± s.d.	*S*-enantiomer mean ± s.d.	*S/R* ratio
Flurbiprofen			
free:	6.03 ± 4.59	5.00 ± 3.86 *	0.83
acyl-conjugate:	5.93 ± 2.02	4.66 ± 1.86 *	0.79
total	11.96 ± 4.23	9.66 ± 3.29 *	0.81
4'-Hydroxyflurbiprofen			
free:	9.23 ± 6.20	7.82 ± 5.07	0.85
acyl-conjugate:	9.73 ± 4.38 †	7.42 ± 3.46 * †	0.76
phenol-conjugate:	1.03 ± 1.01	0.73 ± 0.57	0.71
total	19.99 ± 3.40 †	15.98 ± 2.14 * †	0.80
3'-Hydroxy-4'-methoxyflurbiprofen			
free:	0.016 ± 0.031	0.013 ± 0.027	0.81
acyl-conjugate:	0.011 ± 0.022	0.009 ± 0.018	0.82
phenol-conjugate:	1.51 ± 0.42	1.38 ± 0.31 †	0.91
total	1.53 ± 0.44	1.40 ± 0.31 †	0.91
Total Recovery	33.49 ± 6.32	27.03 ± 4.42 *	0.81

** p-value <0.05 for comparison between the means of enantiomers using Student's t-test for paired samples. † p-value <0.05 for comparison between respective means for young and elderly volunteers using Student's t-test for independent samples.*

Table 6.12: Metabolite formation clearances (ml/min) following the oral administration of racemic flurbiprofen (100 mg) to four healthy elderly volunteers.

(a) Flurbiprofen

Subject	**acyl glucuronide**			**4'-hydroxyflurbiprofen**		
Code	***R***	***S***	***S/R* ratio**	***R***	***S***	***S/R* ratio**
Fe1	1.36	0.83	0.61	4.24	2.89	0.68
Fe2	1.30	0.84	0.65	5.60	4.47	0.80
Fe3	1.87	1.43	0.76	4.45	3.04	0.68
Fe4	1.09	0.78	0.72	6.84	4.69	0.69
Mean	1.41	0.97	0.68	5.28	3.77	0.71
s.d.	0.33	0.31	0.07	1.20	0.94	0.06
C.V. %	23.55	31.73	10.12	22.68	24.88	8.06
p (*R* vs. *S*) *	p<0.005			p<0.01		
p(Y vs. E) †	p < 0.05	p < 0.005	N.S.	p < 0.001	p < 0.0005	N.S.

(b) 4'-Hydroxyflurbiprofen

Subject	**conjugate(s)**			**3'-hydroxy-4'-methoxyflurbiprofen**		
Code	***R***	***S***	***S/R* ratio**	***R***	***S***	***S/R* ratio**
Fe1	25.7	10.9	0.42	4.05	2.03	0.50
Fe2	32.9	21.4	0.65	5.64	3.48	0.62
Fe3	42.0	27.0	0.64	5.29	4.04	0.76
Fe4	32.8	13.9	0.42	4.02	3.04	0.76
Mean	33.4	18.3	0.53	4.75	3.15	0.66
s.d.	6.7	7.3	0.13	0.84	0.85	0.13
C.V. %	20.02	39.92	24.13	17.64	27.01	18.99
p(R vs. S) *	p<0.005			p<0.05		
p(Y vs. E) †	p < 0.01	p < 0.01	N.S.	p < 0.05	p < 0.001	N.S.

** Comparison between the means for the enantiomers were carried out using Student's t-test for paired samples (N.S. = p>0.05).*

† Comparison between respective means for young and elderly volunteers were carried out using Student's t-test for independent samples (N.S. = p>0.05).

Age-associated differences

A lower percentage of the dose, although not statistically significant, was recovered over the 24 hour urine collection period in the elderly (60.5 % vs. 72.3 %) with the overall stereochemical composition of the total material invariant between the two age groups (*S/R*: young = 0.81 and elderly = 0.87; Tables 6.9 and 6.11).

The recovery values of flurbiprofen showed good correlation between the two volunteer groups for both enantiomers (Tables 6.9 and 6.11). However, the proportion of recovered flurbiprofen excreted as the acyl glucuronide is reduced in the elderly group such that it is no longer the primary component; i.e., for the *R*-enantiomer: acyl/free = 2.8 for the young and 0.99 for the elderly, and for the *S*-enantiomer: acyl/free = 2.7 for the young and 0.93 for the elderly.

The total recovery of both enantiomers of the 4'-hydroxy metabolite were significantly decreased in the elderly. This was primarily due to the considerably reduced presence of the acyl glucuronide of (*R*)- and (*S*)-4'-hydroxyflurbiprofen in the recovered material for the elderly group and it is worth noting that the phenol conjugate, although a minor component, was recovered to a similar extent in both age groups. In addition, as observed with flurbiprofen, the quantities of the enantiomers of the 4'-hydroxy metabolite recovered nonconjugated were greater in the elderly group such that elimination of the metabolite in the free form was quantitatively just as important as acyl glucuronidation for this age group, i.e., for the *R*-enantiomer: acyl/free = 3.1 for the young and 1.05 for the elderly, and for the *S*-enantiomer: acyl/free = 2.8 for the young and 0.95 for the elderly.

3'-Hydroxy-4'-methoxyflurbiprofen is a relatively minor metabolite, however an interesting age-related alteration in the enantioselective excretion of this product as its phenol conjugates was detected. The recovery of both (*R*)- and (*S*)-3'-hydroxy-4'-methoxyflurbiprofen phenolic conjugates were lower in the elderly, but the decrease was only significant for the *S*-enantiomer which resulted in a reversal of the prominent enantiomer in the total recovered material, i.e. *S/R*: young = 1.17 and elderly = 0.91 (Tables 6.9 and 6.11).

This pattern of age-related differences in the excretion profile is reflected in the corresponding renal and metabolite formation clearances. Comparison of the renal clearances between the young and elderly for flurbiprofen (*R*: young = 1.2 and elderly = 1.4; *S*: young = 0.9 and elderly = 1.1 ml/min) and for 4'-hydroxyflurbiprofen (*R*: young = 34.5 and elderly = 31.8; *S*: young = 18.4 and elderly = 17.8 ml/min) showed no age-

related differences (Tables 6.5 and 6.6). However, metabolite formation clearances were all significantly reduced in the elderly being between two to three fold less than the corresponding values in the young group (Tables 6.10 and 6.12). It is worth noting that these age-related alterations in urinary excretion and formation clearances are not associated with enantioselective changes since the *S/R* ratios for the various parameters were consistent between the two age groups, this finding mirrors a similar observation with the serum pharmacokinetic data.

6.4. Discussion

Previous studies comparing the pharmacokinetics of flurbiprofen in different age groups are rare and are based on non-stereospecific drug determinations. These investigations were also performed with participants having underlying pathological conditions, and the findings of these studies suggest that age has a negligible influence on the disposition of flurbiprofen (Hamdy *et al.*, 1980; Kean *et al.*, 1992). However, the conclusions drawn by Hamdy *et al.* (1980) may be misleading due to limitations in the experimental design, assessment of age-related alterations in pharmacokinetics were performed in a retrospective manner with the experimental findings compared to those from a previous study using young healthy volunteers. The investigations carried out by Kean *et al.* (1992) were considerably more extensive and included examination of the urinary recoveries of flurbiprofen and its metabolites; nevertheless, the volunteers selected for the younger age group had a average age of 53 years and could be considered to be of semi-advanced age themselves and so the findings may not truly reflect the situation observed with a younger age group.

Enantioselective disposition

The results of the present study suggest that there are age-associated differences in the disposition of flurbiprofen since a greater number of enantioselective differences in the determined pharmacokinetic parameters were observed in the elderly compared to the young volunteers (Tables 6.1 and 6.5). Of significant importance, is the lower area under the curve (AUC) observed for (*R*)-flurbiprofen compared to (*S*)-flurbiprofen in the elderly (*R*: 69.5 vs. *S*: 79.3 μg/ml hr) and the corresponding degree of enantioselectivity

(*S/R* = 1.14) is in agreement with previous studies performed with healthy volunteers (Jamali *et al.*, 1988, 1991; Cefali *et al.*, 1991; Knadler *et al.*, 1992a; Geisslinger *et al.*, 1994b). Such a difference in AUC for a pair of enantiomers following the oral administration of the drug may be due to enantioselectivity in bioavailability, systemic clearance or a combination of both. However, such differences are unlikely to be associated with differences in the systemic availability since absorption of most drugs through the gastrointestinal tract is a passive process governed primarily by the physico-chemical properties of the drug (Rowland and Tozer, 1995) and enantioselective presystemic metabolism is unlikely due to the low clearance of flurbiprofen. This seems a reasonable conclusion since the rapid and total systemic availability of flurbiprofen has been reported previously suggesting complete absorption and insignificant first-pass metabolism (Risdall *et al.*, 1978; Szpunar *et al.*, 1987). Thus the significant difference observed in the clearance of the two drug enantiomers, i.e., the slower clearance of (*S*)-flurbiprofen in comparison to (*R*)-flurbiprofen in the elderly, is responsible for the AUC of the pharmacologically more active *S*-enantiomer exceeding that of its antipode. This, together with the greater volume of distribution of (*S*)-flurbiprofen, is reflected in the significantly longer elimination half-life ($t_{1/2,z}$) of (*S*)-flurbiprofen and the enantiomeric ratio (*S/R* = 1.35) is in good agreement with the corresponding ratio for the AUCs.

In the young, a comparable significant enantiomeric difference was only observed for $t_{1/2,z}$ and it would appear that the disposition of the drug exhibits age-related stereoselectivity. However, AUC and CL followed similar trends in the young volunteers to those observed in the elderly group with (*S*)-flurbiprofen having a larger AUC and lower CL than (*R*)-flurbiprofen and the degree of enantioselectivity for these two parameters were in fact comparable between the two groups (i.e. AUC *S/R*: young = 1.17 and elderly = 1.14; and CL *S/R*: young = 0.86 and elderly = 0.88). It is possible that these differences did not achieve statistical significance in the young as a consequence of the much greater inter-subject variability observed for the *S/R* ratios of AUC and CL in the young (Table 6.2 and 6.6).

The enantioselective serum kinetics of the metabolite, 4'-hydroxyflurbiprofen, has been investigated previously; but data analysis was restricted to the determination of the AUC values for the individual enantiomers (Knadler *et al.*, 1992a). In the current study, urinary recovery data was used to establish the oxidative metabolite fraction (F_{ox}) for each enantiomer, i.e. the proportion of the dose that undergoes oxidation to form the

4'-hydroxy and 3'-hydroxy-4'-methoxy metabolites, which in combination with serum data enabled the determination of a more complete set of pharmacokinetic parameters. It is worth noting that the determination of F_{ox} was based on the recovery of between 50 to 75 % of the administered dose for the volunteers and the assumption is made that any unrecovered dose will be eliminated without influencing the determined F_{ox} value. This is acceptable as preliminary urinary excretion studies performed previously, using three volunteers, demonstrated that the proportion of oxidative metabolites present in recovered material was essentially invariant with increasing urinary collection period (see Chapter 5). Examination of the determined pharmacokinetic parameters revealed the same enantiomeric differences in both the elderly and young age groups (Tables 6.3 and 6.7). The predominance of (*S*)-4'-hydroxyflurbiprofen in serum, as indicated by higher AUC and C_{max} values for the (*S*)-enantiomer in both groups, is predominately as a consequence of the *R*-enantiomer being cleared significantly faster than its antipode in both groups. Stereoselective formation clearance favouring the *R*-enantiomer has a comparatively minor influence on the serum profiles since formation clearances were typical between 14 to 20 fold less than the associated total clearances (Tables 6.3, 6.7, 6.10 and 6.12). These observations are in contrast to those of Knadler *et al.* (1992a) were a lack of stereoselectivity in the disposition of 4'-hydroxyflurbiprofen was suggested based on the insignificant deviation of the *S/R* metabolite AUC ratio from unity.

The above findings indicate that enantiomeric differences in the disposition of flurbiprofen stem predominantly from enantioselectivity in systemic clearance. Like other profen drugs, flurbiprofen is cleared almost exclusively by hepatic metabolism (Williams *et al.*, 1993) and the clearance of such drugs is given by:

$$\mathbf{CL = f_u.CL_u} \qquad \text{(Eqn. 6.8)}$$

where f_u is the fraction of drug unbound in the plasma and CL_u is the unbound or intrinsic metabolic clearance of the drug from plasma (Rowland and Tozer, 1995). Therefore, differences in total clearance between enantiomers may be due either to differences in unbound clearance or in protein binding.

The binding properties of flurbiprofen enantiomers have been investigated using equilibrium dialysis and ultrafiltration in plasma from healthy volunteers (Knadler *et al.*, 1989). Modest stereoselective binding was indicated with the mean percent unbound of

the *R*-enantiomer being greater than that of the *S*-enantiomer, this is consistent with the higher total clearance of (*R*)-flurbiprofen observed in the current investigations. However, inter-individual variation in the enantiomeric ratio (*S/R*) of the free fraction was considerable, varying between 1.92 and 0.75 with a mean of 0.94. Moreover, a recent investigation demonstrated no significant difference in the plasma binding of flurbiprofen enantiomers and the mean enantiomeric ratio (*S/R*) of 1.23 was suggestive of higher free fractions of the *S*-enantiomer rather than its antipode (Blouin *et al.*, 1993). Therefore, in contrast to ibuprofen, the enantioselectivity in plasma protein binding of flurbiprofen is not well defined and inter-individual variations in the enantiomeric ratio may be an important source of variability in the degree of stereoselectivity in disposition which could conceivably be responsible for a reversal in stereoselectivity in some individuals.

To identify the principle pathways of flurbiprofen elimination, urinary recovery studies were performed and formation clearances determined for the metabolites to establish the relative contributions of the pathways to the overall enantioselectivity observed in the total clearance. Examination of the urinary excretion data indicates that the principle metabolites of flurbiprofen are 4'-hydroxyflurbiprofen and the glucuronide conjugate (Tables 6.9 and 6.11). The 4'-hydroxy metabolite is cleared predominantly in the form of the acyl glucuronide and further oxidation and methylation to yield 3'-hydroxy-4'-methoxyflurbiprofen appears to be a comparatively minor elimination route. The preferential elimination of products with the *R*-configuration is reflected in the predominance of "*R* metabolites" recovered in the urine for both age groups; however in the young age group, the 3'-hydroxy-4'-methoxy metabolite, which accounts for only 4 % of the dose, is excreted to a larger, but not significant, extent in the *S*-form.

The formation clearance associated with oxidation to 4'-hydroxyflurbiprofen, as would be expected, shows substrate enantioselectivity favouring (*R*)-flurbiprofen in both the young and elderly groups (Tables 6.10 and 6.12). Such differences could clearly be caused by inherent stereoselectivity in functional oxidation, however this appears unlikely in this case since the mean *S/R* ratio for the oxidative metabolite fraction (F_{ox}) were equal to unity for both age groups (Tables 6.2 and 6.6). Furthermore, *in vitro* experiments carried out in order to characterise the kinetic parameters for the oxidation of (*R*)- and (*S*)-flurbiprofen, using human liver microsomes, highlighted a lack of stereoselectivity (Tracy *et al.*, 1995). These studies, indicated that the enantiomers of flurbiprofen may exhibit stereoselectivity with respect to enzyme affinity (K_m, *R*: 3.1 vs.

S: 1.9 μM) but have similar maximum formation velocities (V_{max}, *R*: 305 vs. *S*: 343 pmol/min/mg protein) and with the racemate, enantiomer-enantiomer interactions results in decreased K_m and V_{max} for both enantiomers and an apparent loss of stereoselectivity.

The formation clearances of flurbiprofen acyl glucuronide and 4'-hydroxy-flurbiprofen acyl glucuronide also displayed a preference for the *R*-enantiomers (Tables 6.10 and 6.12). These findings show good correlation with *in vitro* data that indicates glucuronidation of flurbiprofen favours the *R*-enantiomer (Hamdoune *et al.*, 1995). However, these findings must be interpreted with caution since it is also clear that enantioselective hydrolysis of acyl glucuronides may occur in tissue, plasma or both (Faed, 1984; Knadler and Hall, 1991). The formation clearances associated with acyl glucuronides must, therefore, be considered as net clearances reflecting a balance between formation and hydrolysis, which may in turn reflect the rate of elimination of the conjugate (Faed, 1984).

However, having said that, the degree of enantioselectivity (i.e. *S/R* ratio) showed very little difference between the clearance of flurbiprofen via glucuronidation or oxidation in both age groups (Tables 6.10 and 6.12). This would suggest that modest or no enantioselectivity is observed in the metabolic processes and that enantioselectivity in protein binding, favouring a higher free fraction of (*R*)-flurbiprofen, is of greater significance. In contrast with 4'-hydroxyflurbiprofen, greater enantioselectivity was observed for elimination of the metabolite via conjugation than through further oxidation. This could possibly be as a consequence of a more prominent substrate enantioselectivity for the formation of the acyl glucuronide of the *R*-enantiomer being observed with 4'-hydroxyflurbiprofen than the parent drug.

Age-associated differences

Major age-related differences in the disposition of flurbiprofen appear to originate from a general reduction in clearance of the drug in the elderly and are observed with both enantiomers. The systemic clearances of (*R*)- and (*S*)-flurbiprofen in the elderly are 62 % and 63 % respectively of the corresponding clearances in the young, and consequently the elderly have significant higher values for AUC (*R*: 69.5 vs. 42.3; *S*: 79.3 vs. 49.5 μg/ml hr) and terminal elimination half lives for both enantiomers (*R*: 7.1 vs. 4.5; *S*: 9.6 vs. 5.2 hr; Tables 6.1 and 6.5). Similar age-related differences were

observed in the serum kinetics of the enantiomers of 4'-hydroxyflurbiprofen, the major metabolite (Tables 6.3 and 6.7).

Such differences in the disposition of a low extraction drug such as flurbiprofen could be clearly be associated with age-related alterations in protein binding; however, *in vitro* studies have shown that the plasma protein binding of racemic flurbiprofen in elderly volunteers (≥ 65 years old) is not significantly different from normal subjects (Knadler *et al.*, 1989). Moreover, if any age-related alterations in protein binding do occur then unbound fractions are likely to increase rather than decrease, with or without associated changes in stereoselectivity, since increasing age is associated with a decrease in serum albumin concentration (Wallace and Verbeeck, 1987; Woodhouse and Wynne, 1987).

It is therefore likely that age-related changes in other factors alternate to protein binding, such as in renal function and/or drug metabolising capacity of the liver, play a more prominent role in the observed differences in flurbiprofen disposition between the two age groups. The reduced urinary recoveries of the total oxidative and conjugated metabolites in the elderly, but with concurrent higher recoveries of flurbiprofen and 4'-hydroxyflurbiprofen in the free form suggest that reduced metabolic activity rather than a general decrease in renal elimination is the principle cause (Tables 6.9 and 6.11). Furthermore, the decreased elimination via oxidation and conjugation pathways is reflected in all the metabolite formation clearances being significantly lower in the elderly group (Tables 6.10 and 6.12). It is also worth noting that renal clearances of the enantiomers of flurbiprofen and 4'-hydroxyflurbiprofen displayed no age-related alterations (Tables 6.1,6.3,6.5 and 6.7).

The affect of age on the drug metabolising capability of the liver is not well defined and the subject of much contention (Woodhouse and Wynne, 1987). The major physiological changes that occur with age which may influence hepatic drug elimination include an absolute (and relative to body weight) decrease in the size of liver and reduced regional blood flow to this organ (Kinirons and Crome, 1997). Results from *in vitro* studies have indicated that flurbiprofen oxidation to 4'-hydroxyflurbiprofen is predominately mediated by cytochrome P450 2C9 (CYP 2C9) (Tracy *et al.*, 1996) and age related studies have indicated that generally clearance via CYP mediated metabolism is reduced in the elderly, which is consistent with our findings (Sotaniemi *et al.* 1997, Hammerlein *et al.*, 1998). However, the effect of age on phase II metabolic reactions such as glucuronidation and methylation has not been the subject of extensive

research, but it has been suggested that glucuronidation is not generally affected by ageing (Mooney *et al.*, 1985). The decreased clearance of ketoprofen in elderly volunteers, a drug principally eliminated via the formation of an acyl glucuronide, has been reported previously (Advenier *et al.*, 1983; Dennis *et al.*, 1985). Although, whether such changes were associated with decreased glucuronidation were not established.

The decreased elimination of flurbiprofen via the oxidation and acyl glucuronidation pathways in the elderly appear not to be associated with stereoselective changes as indicated by the invariance of corresponding *S/R* ratios for metabolites recoveries between the two groups. This suggests that the oxidation and glucuronidation reactions are most probably carried out using single respective isoenzymes and any possible age-related alteration in protein binding is not expected to show stereoselectivity. However, in the case of the minor metabolite, 3'-hydroxy-4'-methoxyflurbiprofen, there appears to be a reversal in the stereoselectivity of elimination with age. i.e. the 3'-hydroxy-4'-methoxy metabolite is predominately excreted as phenolic conjugates of the *S*-enantiomer in the young and the *R*-enantiomer in the elderly. It is possible that phenolic conjugation of 3'-hydroxy-4'-methoxy-flurbiprofen to form either ether glucuronides and/or sulphate conjugates display opposing enantioselectivity such that the reduction in clearance with age is also associated with a change in the ratio of conjugation via the two pathways which results in a reversal in overall conjugation stereoselectivity.

6.5. Conclusions

Modest stereoselective differences were observed in the pharmacokinetics of the enantiomers of flurbiprofen following the administration of the racemic drug to healthy elderly volunteers. Enantiomeric differences in total clearance, favouring the faster elimination of the *R*-enantiomer (*S/R* = 0.88), resulted in higher values for AUC and $t_{1/2,z}$ for the pharmacologically more potent (*S*)-flurbiprofen. In the young group, a similar statistical significant difference was seen for the $t_{1/2,z}$, and comparable trends in AUC and CL were observed but did not reach statistical significance as a result of the greater inter-subject variation in enantioselectivity for these parameters. The primary pathway for flurbiprofen elimination is oxidation to form 4'-hydroxyflurbiprofen which accounted for about 36 % and 47 % of the recovered dose in the elderly and young

groups respectively. The same enantioselective differences in the serum kinetics of this metabolite were noticed in both age groups. Like the parent compound, enantioselective clearance of (*R*)-4'-hydroxyflurbiprofen was primarily responsible for the differences observed in AUC, $t_{1/2,z}$ and C_{max} between the enantiomers. As would be expected there was a predominance of products with the *R*-configuration in urine and this pattern is reflected in the metabolic formation clearances which all displayed substrate stereoselectivity in favour of the *R*-enantiomer.

Age-related differences were observed in the disposition of flurbiprofen largely as a consequence of the reduced clearance of the drug in the elderly. The reduced clearance is not associated with enantioselective alterations and thus similar age-related differences are observed for both enantiomers. Age-related decrease in hepatic function appears to be the primary cause resulting in reduced elimination via metabolism. The major outcome is that the elderly have greater exposure to the drug, as reflected by the AUCs for the enantiomers of flurbiprofen being approximately 60 % higher in this age group in comparison to the young; such differences could have major pharmacological and toxicological implications for the use of the drug in the elderly.

CHAPTER 7 :

General discussion and conclusions

7.1. Introduction

The prevalence of rheumatic diseases increases progressively with advancing age. Data from an interview survey indicate that self-reporting of arthritis by individuals living in the community increases from 337 per 1,000 among those aged between 55 to 64 to nearly 600 per 1,000 for those aged 85 and older (Cohen and von Nostrand, 1995). Non steroidal anti-inflammatory drugs (NSAIDs) remain the mainstay of treatment for many rheumatic conditions, and the use of these agents is greatest in the elderly population; it has been estimated that 10% to 15% of those aged 65 and older take prescribed NSAIDs (Ray *et al.*, 1993) and there may be a similar, if not greater, percentage using these medications "over the counter".

Of increasing concern in recent years has been the adverse effects associated with the use of NSAIDs and this has been the subject of numerous epidemiological studies. A recent extensive meta-analysis, combining data from 49 different studies, indicated that on average 1 in 1200 patients taking NSAIDs for at least two months will die from gastrointestinal complications who would not have died had they not taken NSAIDs (Tramèr *et al.*, 2000). These calculations clearly suggest that chronic oral NSAIDs pose a substantial risk of death from gastrointestinal complications and could be responsible for 2000 deaths each year in the UK (Tramèr *et al.*, 2000). It is also generally accepted that the elderly are more prone than younger patients to the toxic effects of NSAIDs (Henry *et al.*, 1993; Garcia Rodriguez and Jick, 1994; Wolfe *et al.*, 1999), although few data exists to suggest that advanced age is an independent risk factor. However, the severity of the problem, together with its associated costs, has been highlighted in the study carried out by Cunningham *et al.* (1997), which demonstrated that NSAIDs were responsible for 28% of all drug related problems requiring hospitalisation of elderly people over a 9-month study period.

The studies described in this thesis were stimulated by the following question: "Are the elderly at greater risk of NSAID-related adverse effects due to alterations in the disposition of NSAIDs in this population?". Surprising there has been a scarcity of dispositional studies on NSAIDs designed to examine the influence of ageing and the limited data available is plagued by inconsistent findings. Furthermore, the majority of these studies have been of limited scope and not attempted to obtain a comprehensive picture of the disposition of the NSAID via different pathways or considered stereochemical aspects in the case of chiral NSAIDs. Ibuprofen and flurbiprofen are two

important chiral 2-arylpropionic acid derivatives used clinically as NSAIDs almost exclusively in their racemic form. Following the development and validation of appropriate stereospecific methodologies for the bioanalysis of these two NSAIDs and their major metabolites, the enantioselective disposition of the racemic drugs were investigated in the young and elderly to gain a clearer understanding of the influence of ageing.

7.2. Stereospecific bioanalysis

The various stereospecific analytical methods developed for the quantitative analysis of ibuprofen, flurbiprofen and their metabolites can be broadly categorised into two different chromatographic approaches: (a) indirect separation on an achiral reversed phase system following pre-column derivatization with the homo-chiral derivatizing reagent (HCDA), (*R*)-1-(naphthen-1-yl)ethylamine (NEA); or (b) direct separation on a polysaccharide CSP, i.e either cellulose tris(3,5-dimethyl phenylcarbamate) (Chiralcel OD) or amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak AD), via normal phase HPLC.

7.2.1 Indirect approach

The indirect approach was adopted for the analysis of ibuprofen enantiomers in serum and urine, with a conventional, cheap and highly efficient reversed phase C_{18} column used for the resolution of the fluorescent diastereomeric amides formed on reaction with (*R*)-NEA (Chapter 2). Derivatization was performed using CDI and HOBT as coupling reagents; which although required relatively long reaction times, was essentially quantitative and did not pose racemisation problems like those encountered with the mixed anhydride derivatization method (Hutt *et al.*, 1994).

The commercial availability of both enantiomers of NEA in high enantiomeric purity offers the flexibility to control the order of elution. In a reversed phase system, using (*R*)-NEA as the HCDA results in the diastereomeric amide derivative of (*R*)-ibuprofen eluting before that of (*S*)-ibuprofen and when (*S*)-NEA is used the elution order is reversed. For the assay methods, (*R*)-NEA was selected as the HCDA so that the (*R*)-ibuprofen derivative eluted first since (*R*)-ibuprofen concentrations were expected to be lower than those of the *S*-enantiomer in serum, and earlier elution would reduce the

effects of band broadening on a smaller peak and hence increase analytical sensitivity. It is also noteworthy, that under normal phase conditions, the order of elution is again the opposite of that for the reversed phase system (Hutt *et al.*, 1994; Tan *et al.*, 1999).

A major drawback of the indirect approach, especially if a strong fluorophore or chromophore such as (*R*)-NEA is used, is that the excess chiral amine when injected onto the HPLC column will result in a large reagent peak that inevitably causes interference problems. Therefore a silica solid-phase extraction procedure was employed after derivatization, with the acidic silanol groups scavenging the unreacted amine whilst allowing the passage of the neutral diastereomeric amides.

On the other hand, the fluorogenic properties of the naphthyl moiety allows for the use of a fluorescence detector and thus affords additional sensitivity. Comparison of the sensitivity, in terms of the minimal quantifiable limit, with those previously reported; indicates that the assay method used is more sensitive than those bioanalytical HPLC procedures based on CSPs (Nicoll-Griffith *et al.*, 1988; Geisslinger *et al.*, 1989; Li *et al.*, 1993; Naidong and Lee, 1994; de Vries *et al.*, 1994), equally if not more sensitive than those based on indirect approaches with UV detection (Lee *et al.*, 1984; Avgerinos and Hutt, 1987; Mehvar *et al.*, 1988) and equally sensitive with the methods based on fluorescence detection (Lemko *et al.*, 1993; Lau, 1996). Furthermore, the comparatively long analysis time of the method used, is compensated by superior resolution characteristics and less peak interference from co-extracted endogenous substances than was evident for the methods cited in the literature.

The general applicability of the approach for the indirect resolution of other chiral 2-arylpropionic acids was also suggested. Using the same column and with adjustments in the mobile phase flow rate, the (*R*)-1-(naphthen-1-yl)ethylamide diastereomeric pairs for flurbiprofen and it's two major metabolites, 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen, could all be successfully baseline resolved as indicated by resolution factors greater than 2.0 (Chapter 4). In addition, a modest modification of the mobile phase composition would have allowed all three compounds to be analysed in a single chromatographic run. However, this was not attempted since the use of fluorescence detection exposed a major disadvantage of the indirect approach with the pair of derivatives formed for each analyte, in this case, displaying differential detector responses. The significant nature of the differences, with the peak area ratios for the diastereomeric pairs ranging from 0.35 to 0.82, rendered the approach inappropriate for application in quantitative analysis.

7.2.2 Direct approach

The polysaccharide CSPs based on the tris(3,5-dimethylphenylcarbamate) derivative of cellulose (Chiralcel OD) and amylose (Chiralpak AD) show excellent enantioselectivity and have the ability to resolve a wide range of racemates having a variety of functional groups (Yashima and Okamato, 1997; Okamato and Yashmima, 1998). However, their application in bioanalytical studies have been rather restricted because the columns are used under normal phase condition which necessitates the transfer of the analytes from the aqueous biological matrix to an organic phase prior to analysis. The presence of trace amounts of water may have detrimental effects by swelling or dissolving the polysaccharide-coat and any co-extracted endogenous material, which is likely to be strongly retained on the silica support, may cause even further damage. Thus for routine use in bioanalysis, the sample work-up procedure is critical to ensure the long-term stability, efficiency and selectivity of these costly CSP columns.

Previous investigations on the enantiomeric resolution of 2-arylpropionic acids using Chiralcel OD and Chiralpak AD CSPs indicate that they have different chiral recognition properties since ketoprofen, flurbiprofen and tiaprofenic acid were only resolved on the Chiralpak AD column (Okamoto *et al.*, 1989). Consistent with such observations, was our ability to resolve the enantiomers of ibuprofen successfully using the Chiralcel OD column (Chapter 2) but not with the Chiralpak AD phase (unpublished observations). Okamoto and Yashmina (1998) attributed the difference in chiral recognition ability between the two CSPs to the differences in their higher order structure. Possible structures are left-handed 3/2 and 4/1 helical chain conformations for tris(3,5-dimethylphenylcarbamates) of cellulose and amylose respectively (Okamoto and Yashmina, 1998).

The direct resolution of ibuprofen enantiomers on the Chiralcel OD column was applied in methodology, using equilibrium dialysis and radiolabelled racemic ibuprofen, for the determination of unbound enantiomer concentrations in serum. Essentially, the individual enantiomer eluate fractions were collected, following isolation of ibuprofen from post-dialysis serum or buffer, for radiochemical analysis. Sample preparation was a simple one-step extraction procedure, which was deemed acceptable since only small aliquots of serum were used and the buffer samples, although of larger volume, were expected to be relatively free of contaminants. The use of radiolabelled drug is essential for the sensitive detection of enantiomeric differences at extremely low concentrations

and by using the direct chromatographic separation procedure, sample manipulation is considerably less than in previous methods employing the indirect approach (Evans *et al.*, 1989; Tan *et al.*, 1997a) and thus analyte loss and potential sources of error are kept to a minimum.

In contrast to the parent drug, baseline resolution of both enantiomers of hydroxyibuprofen and for all four stereoisomers of carboxyibuprofen was achievable by use of the Chiralpak AD CSP (Chapter 2). However, the stereospecific analysis of these metabolites in urine was performed using a sequential achiral-chiral chromatographic approach to ensure that only "clean" samples were introduced into the Chiralpak AD column. The additional chromatographic step thus eliminated the need for a sophisticated sample purification procedure. This assay method had lower limits of quantification than the other reported chromatographic method, which was based on the indirect approach requiring the formation of (*S*)-1-phenylethylamide derivatives (Rudy *et al.*, 1990). The throughput of the procedure could potentially be improved by column coupling and on line automation. With the use of a switching valve, the eluate fractions from the normal phase column containing the analytes can be diverted onto the CSP without detriment to chromatographic resolution since the mobile phases are similar, the only difference is that the proportion of ethanol in the mobile phase increases from 1.8% v/v to 8% v/v. Hence, diverting 1-2 ml of the achiral mobile phase onto the CSP should not influence resolution as there would be a concentration effect on the chiral column resulting in peak sharpening.

The successful resolution of the enantiomers of 4'-hydroxyflurbiprofen and 3'-hydroxy-4'-methoxyflurbiprofen on the Chiralpak AD CSP is a further indication of its versatility (Chapter 4). Unlike alternative CSPs such as those based on proteins, polysaccharide columns have a relatively high loading capacity so that large amounts of material could be injected on-column without appreciable loss of resolution. This coupled with the better than baseline resolution for these two flurbiprofen metabolites enabled on-column injections of up to 400 μg of racemic material. In this manner milligram quantities of the individual enantiomers could be isolated from the analytical column which allowed the chiroptical properties of the individual enantiomers of both compounds to be recorded for the first time. However, the assignment of stereochemical configurations using circular dichroism proved be more difficult than previously experienced with other 2-arylpropionic acids (Tan *et al.*, 1997c; Hoult *et al.*, 1999). This was primarily due to interference of the electronic transitions of the carboxyl group by

biphenyl-related signals, with the composite nature of the CD spectra further complicated by the influence of solvent and substitution on the biphenyl ring system. The assigned stereochemistry, which indicated that the elution order on the CSP was *R*- before the *S*-enantiomer for both metabolites, was subsequently confirmed by the analysis of urine extracts following the administration of the individual enantiomers of flurbiprofen to a healthy volunteer.

The ability to separate and resolve the enantiomers of these metabolites and those of flurbiprofen together on the Chiralpak AD CSP within an acceptable run time, was appropriate for adaptation into an analytical method for the determination of both the drug and two major metabolites in urine (Chapter 5). To ensure the practicality of the approach for routine bioanalysis, a one-step extraction procedure was utilised which compromised optimal analyte recoveries to assure the CSP was exposed to "contaminant-free" extracts. Furthermore, simultaneous quantification of the analytes over differing concentration ranges was only made possible by the use of a dual, UV and fluorescence, detection method. A more sophisticated approach was also necessitated to quantify the conjugates than was used in the urine assay methods for ibuprofen and its metabolites, since the oxidative metabolites of flurbiprofen undergo conjugation of the phenolic hydroxyl in addition to acyl glucuronidation. For this purpose, an enzymatic-base hydrolysis combination method was employed, in addition to a base hydrolysis procedure. The serum assay method was confined to the analysis of the enantiomers of flurbiprofen and the 4'-hydroxy metabolite and so only required the use of fluorescence detection. However, the presence of interfering material in volunteer serum extracts, which was most likely to be drug-related, necessitated alteration in the mobile phase composition and the use of an alternative internal standard.

7.3. Stereoselective disposition of ibuprofen and flurbiprofen

Both ibuprofen and flurbiprofen demonstrated enantioselective disposition in humans, with the major enantiomeric differences for each drug evident in both the young and elderly (Chapters 3 and 6). However, comparison of the pharmacokinetic parameters of the two drugs clearly indicates that enantioselectivity in disposition is more apparent for ibuprofen than for flurbiprofen (Table 7.1). These differences in

enantiomeric disposition are primarily associated with enantioselectivity in plasma protein binding and/or metabolism of the drug.

Table 7.1: Pharmacokinetic parameter ratios (*S/R*) based on total serum concentrations, following the oral administration of racemic ibuprofen (400 mg) or racemic flurbiprofen (100 mg) to healthy young volunteers [†].

Parameters	Enantiomeric ratio (*S/R*)	
	ibuprofen (n=8)	**flurbiprofen (n=4)**
C_{max}	0.99	0.96
T_{max}	1.12	1.46
$t_{1/2,z}$	1.50 *	1.15 *
AUC	1.28 *	1.17
CL	1.27 *	0.86
V_d	2.04 *	0.99
% Unbound	2.09 *	N.D.

** p-value <0.05 for comparison between the means of enantiomers using Student's t-test for paired samples.*

[†] N.D. = not determined.

7.3.1 Plasma protein binding

The plasma protein binding of ibuprofen was shown to be extensive and stereoselective in favour of the *R*-enantiomer, with percentage unbound fractions of 0.22% and 0.46% for (*R*)- and (*S*)-ibuprofen respectively following the administration of the racemic drug to young volunteers. The confinement of ibuprofen to the systemic circulation due to its high plasma protein binding results in typically low values for volume of distribution (V_d) for the enantiomers. The significantly greater apparent V_d for (*S*)-ibuprofen is indicative of its higher free levels; this enantioselectivity is lost when volume of distributions are based on unbound serum concentrations (V_{du}) (Chapter 3). A characteristic feature of most NSAIDs is that they have a low hepatic extraction ratio, that is the intrinsic clearance of the drug is small relative to hepatic blood flow and only the unbound drug presented to the liver is available for clearance (Lin *et al.*, 1987). Thus clearance of each ibuprofen enantiomer is directly proportional to their respective free fractions in the plasma, and so (*S*)-ibuprofen having a greater clearance than the *R*-antipode is primarily reflective of its lower association with plasma

protein. This central importance of protein binding is further emphasised when it is considered that unbound clearance displays opposite enantioselectivity to total clearance, i.e. shows preference for the *R*-enantiomer (Chapter 3).

Investigations into the stereoselective protein binding of flurbiprofen were not performed during the course of these studies due to the non-availability of a radiolabelled form of the drug. The development of a method based on an alternative, non-radiochemical, detection approach was deemed unlikely to afford an acceptable degree of sensitivity, considering the serum concentrations of flurbiprofen were comparatively low due to the smaller administered dose, e.g. C_{max} values for flurbiprofen enantiomers were approximately half those of the ibuprofen enantiomers. Previous, *in-vivo* studies have highlighted the extensive nature of the protein binding of flurbiprofen but painted an inconsistent picture with regards to enantioselectivity, each report suggesting modest preference for the opposing enantiomer (Knadler *et al.*, 1989; Blouin *et al.*, 1993). However, *ex vivo* determinations for healthy volunteers and ureamic patients both indicate that (*S*)-flurbiprofen is more tightly bound, with a mean enantiomeric ratio (*S/R*) of the free fraction of 0.87 and 0.81 in the two groups respectively (Knadler *et al.*, 1992a, 1992b). It is noteworthy, that the enantiomeric ratios (*S/R*) for total clearance, having values of 0.86 and 0.88 for the young and elderly volunteers respectively in the present study, display close correlation to such degrees of enantioselectivity in protein binding. Therefore in comparison to ibuprofen, it would appear that enantioselectivity in protein binding of flurbiprofen is more modest and favours the opposite enantiomer of the drug. The limited difference in the protein binding of the flurbiprofen enantiomers is further suggested by the closeness of the enantiomeric ratio of V_d, based on total concentrations, to unity (Table 7.1).

7.3.2 Metabolism

A major difference in the disposition of the two drugs in humans, and primarily responsible for ibuprofen exhibiting greater enantioselectivity in its pharmacokinetics, is the metabolic inversion of (*R*)-ibuprofen to (*S*)-ibuprofen. The administration of the individual enantiomers of flurbiprofen to a volunteer during the preliminary investigations reported within this thesis did not result in the formation of their antipodes, indicating that chiral inversion of flurbiprofen does not occur to significant extent (Chapter 5). An observation in agreement with data reported in the literature (Jamali *et al.*, 1988; Geisslinger *et al.*, 1994b). In contrast, the significance role played

by the chiral inversion reaction in the disposition of ibuprofen can be appreciated when it is considered that the unbound clearance of (*R*)-ibuprofen via inversion was approximately two-fold that by non-inversion pathways and equal to the unbound clearance of (*S*)-ibuprofen (Chapter 3).

Like most 2-arylpropionic acid drugs, ibuprofen and flurbiprofen are excreted predominately in urine and mainly in the form of metabolites, i.e. the liver is the major organ involved in the elimination of the parent drug, while the kidneys are primarily responsible for the excretion of the products (Table 7.2). A common metabolic pathway for both drugs is conjugation of the carboxylic acid functional group to form acyl glucuronides. However, quantitatively the major route of elimination is oxidation; ibuprofen is metabolised in the *iso*-butyl side group to form hydroxy- and carboxyibuprofen and flurbiprofen undergoes aromatic oxidation to produce 4'-hydroxyflurbiprofen. These oxidative metabolites also undergo phase II metabolism and all display a preference for glucuronic acid conjugation in the propionic acid side chain rather than conjugation at the introduced functional group. The 4'-hydroxy metabolite of flurbiprofen also undergoes further oxidation followed by methylation to yield 3'-hydroxy-4'-methoxyflurbiprofen, although this is only a minor elimination pathway (Table 7.2).

Clearance of ibuprofen via the oxidation and glucuronidation metabolic pathways, exhibited substrate selectivity in favour of the *S*-enantiomer which was as result not only of the stereoselective protein binding but also inherent stereoselectivity in the metabolic pathways. The degree of enantioselectivity was quite varied between different metabolic pathways with the most marked effect noted for the formation of ibuprofen glucuronides (Table 7.2). Stereoselective clearance of flurbiprofen via oxidation and glucuronidation was also evident; however in this case, the enantiomeric differences were relatively modest and preference was for the *R*-enantiomer of the drug rather than its *S*-antipode. However, the magnitude of the enantioselectivity was not dissimilar between the two pathways and was comparable to the enantioselective ratios for the unbound fractions quoted above from previous dispositional studies. Therefore, it would appear that the *in vivo* metabolic fate of flurbiprofen, in contrast to ibuprofen, is not associated with significant enantioselectivity at the level of enzymatic oxidation and glucuronidation. The lack of enantiomeric differences in the oxidation of flurbiprofen is further suggested

by the relatively small deviation of the mean *S/R* ratio for the oxidative metabolite fraction (F_{ox}) from unity in both age groups (Chapter 6). Furthermore, *in vitro* investigations of the oxidative metabolism of flurbiprofen enantiomers are in agreement with these findings, suggesting a lack of stereoselectivity (Tracy *et al.*, 1995). Similar *in vitro* studies of the glucuronidation of flurbiprofen, however, do suggest that the conjugation pathway is stereoselective in humans (Hamdoune *et al.*, 1995); but application of such information to the clinical situation must be done with caution due to the unstable nature of the formed acyl glucuronides and thus the possible significance of stereoselective *in vivo* hydrolysis (Knadler and Hall, 1991).

Table 7.2: Urinary excretion data (0-24 hr) following the oral administration of racemic ibuprofen (400 mg) or racemic flurbiprofen (100 mg) to healthy young volunteers.

	Urinary recovery (0-24 hr)	
Metabolite	**% of the dose**	**ratio (*S/R*)**
Ibuprofen (n=8)		
free	1.02	15.5 *
acyl glucuronide	11.1	11.5 *
hydroxyibuprofen †	21.2	5.4 *
carboxyibuprofen †	39.4	4.3 *
Total	**72.7**	**5.2** *
Flurbiprofen (n=4)		
free	5.7	0.89 *
acyl glucuronide	15.7	0.86 *
4'-hydroxyflurbiprofen †	46.8	0.85 *
3'-hydroxy-4'-methoxyflurbiprofen †	4.2	1.17
Total	**72.4**	**0.87** *

** p-value <0.05 for comparison between the means of enantiomers using Student's t-test for paired samples*

† total metabolite quantities, i.e. free plus conjugated.

7.4. Age-related alterations in the disposition of ibuprofen and flurbiprofen

Ageing is a natural phenomenon associated with changes occurring at the basic cellular level, leading to altered physiological responses and a general decline in the function of major organs. These changes will affect the ability of the body to handle administered drugs and thus have the potential to cause alterations in both the pharmacokinetic and pharmacodynamic profiles of the drug in the elderly. Some of the physiological changes produced by ageing which may have important implications for altered pharmacokinetics are presented in Table 7.3.

Table 7.3: Age-related physiological changes which have the potential to influence pharmacokinetic parameters.

process	factor	effect
Absorption	gastic acid production	reduced
	gastrointestinal blood flow	reduced
	gastrointestinal motility	reduced
	gastric emptying rate	reduced
Distribution	body mass	reduced
	proportion of body fat	increased
	proportion of body water	reduced
	plasma albumin	reduced
	α_1-acid glycoprotein	increased
Metabolism	liver mass	reduced
	hepatic blood flow	reduced
	hepatic metabolic capacity	reduced
Excretion	glomerular filteration rate	reduced
	renal plasma flow	reduced
	renal tubular secretion	reduced
	filteration fraction	increased

(Adapted from Cromarty, 1985; Dawling and Crome, 1989; Hammerlein et al., 1998)

Advancing age appears to have an influence on the disposition of both ibuprofen and flurbiprofen; however, the nature of the changes are considerably different between the two drugs (Table 7.4). With regard to ibuprofen, age-related differences were only evident for the *S*-enantiomer. In comparison, ageing had an effect on both flurbiprofen enantiomers with the alterations being more marked than for (*S*)-ibuprofen, the extent of the changes were indifferent between the two enantiomers indicating that ageing has no influence on the enantioselectivity of flurbiprofen disposition (Table 7.4). As would be expected, of all the factors listed in Table 7.3, altered serum albumin binding and metabolic activity are the primary determinants of these age-related changes in drug disposition.

Table 7.4: Effect of age on pharmacokinetic parameters, based on total serum concentrations, following the oral administration of racemic ibuprofen (400 mg) or racemic flurbiprofen (100 mg) to healthy young and elderly volunteers [†].

Parameters	Ratio (elderly/young)			
	ibuprofen		flurbiprofen	
	R-isomer	*S*-isomer	*R*-isomer	*S*-isomer
C_{max}	1.03	0.88 *	1.21	1.20
T_{max}	0.69	0.62	1.44	0.96
$t_{1/2,z}$	1.13	1.38 *	1.58 *	1.85 *
AUC	1.25	1.07	1.64 *	1.60 *
CL	0.81	0.96	0.62 *	0.63 *
V_d	0.92	1.28	0.97	1.17
% Unbound	1.00	1.15 *	N.D.	N.D.

** p-value <0.05 for comparison between respective means for young and elderly volunteers using Student's t-test for indepedent samples.*

[†] n = 8 + 8 for ibuprofen and 4 + 4 for flurbiprofen; N.D. = not determined.

7.4.1 Plasma protein binding

Ageing was associated with a significant stereoselective displacement of ibuprofen from binding sites on plasma protein leading to a 15% increase in the unbound fraction of the *S*-enantiomer with no concomitant change in the binding of the *R*-antipode (Table 7.4). In contrast, the binding of racemic flurbiprofen to plasma protein has been shown to be indifferent between healthy young and elderly volunteers (Knadler *et al.*, 1989). The lack of significant age-related alterations in the free fraction

of flurbiprofen is also indicated in the present study by the comparatively minor differences in the V_d for the enantiomers between the two age groups. The observation that clearances were reduced to a similar extent for both enantiomers of flurbiprofen, rather than increased, in the elderly is further supportive of a lack of significant age related alteration in binding since any decrease in the serum levels of albumin with age is more likely to encourage higher free fractions and greater clearance of the drug. It is not surprising that the binding characteristics of flurbiprofen are not altered with age, since it has greater affinity for serum albumin, which is apparent when it is considered that unbound fractions of flurbiprofen are typically one order of magnitude less than those of ibuprofen (Evans *et al.*, 1989; Knadler *et al.*, 1989).

7.4.2 Metabolism

As ageing is associated with decreased liver perfusion and mass, hepatic metabolism would be expected to be reduced, this is reflected by the diminished clearance of both ibuprofen and flurbiprofen via oxidative pathways in the elderly. For ibuprofen, the impaired oxidative activity appeared to be stereoselective with the more prominent reductions evident for (*S*)-ibuprofen, such that the unbound formation clearances for the hydroxy- and carboxy- metabolites derived from this enantiomer were lower by 21% and 24% respectively in the elderly compared to the young (Chapter 3). Whereas in the case of the *R*-enantiomer, the unbound clearances for the hydroxy- and carboxy metabolites were reduced by only 15% and 8% respectively with advancing age (Chapter 3). Interestingly bienzymatic catalysis of ibuprofen oxidation has been suggested with CYP2C8 playing a minor, but significant, role alongside CYP2C9 (Hamman *et al.*, 1997). However, CYP2C8 shows opposing enantioselectivity to CYP2C9, favouring oxidation of (*R*)-ibuprofen, and thus it is possible that the age-related alterations are reflective of a comparatively greater decrease in CYP2C9 activity. Such alterations may have contributed to the overall unbound clearance of (*S*)-ibuprofen being 17 % lower in the elderly. With regards to flurbiprofen, the decrease in oxidative metabolism with age was more extensive than observed with ibuprofen and both enantiomers of the drug were equally effected. This is apparent when it is considered that the formation clearances of (*R*)- and (*S*)-4'-hydroxyflurbiprofen were reduced by 51% and 53% respectively in the elderly. *In vitro* investigations propose that CYP2C9 may be the only isoenzyme involved to any substantial degree in flurbiprofen oxidation and it has been advocated that this reaction maybe a useful *in vivo* and *in vitro* probe for

this particular CYP isoenzyme (Tracy *et al.*, 1996). Such a "metabolic burden" on a single enzyme is indicative of the dramatic and non-stereoselective nature of the changes in oxidative metabolism of flurbiprofen with age.

It is also important to note that polymorphisms in the coding region of the CYP2C9 gene produce variants at amino acid residues 144 (Arg144Cys) and 359 (Ile359Leu) of the CYP2C9 protein which can lead to differences in catalytic properties. For example, (*S*)-warfarin 7-hydroxylation, tolbutamide methylhydroxylation, and phenytoin 4'-hydroxylation are less efficiently catalysed by the Arg 144 → Cys 144 variant of CYP2C9 (Miners and Birkett, 1998). Similarly, the Ile 359 → Leu 359 variant exhibits reduced activity towards CYP2C9 substrates *in vitro* and *in vivo* where less than 1% of Caucasians, Chinese and African-Americans are homozygous at this gene locus and correspond to the tolbutamide poor metaboliser phenotype (Miners and Birkett, 1998). Approximately 20% of Caucasians are heterozygous for the mutation corresponding to the Cys144 variant of CYP2C9, but it is absent from Chinese individuals, and, therefore, the potential for significant interethnic differences in CYP2C9 activity exists (Miners and Birkett, 1998). Recently, it has been demonstrated that the Cys144 variant of CYP2C9 displays intrinsic clearance values for ibuprofen hydroxylations markedly lower than those of the wild-type enzyme but exhibits comparable sulphaphenazole K_i values (Hamman *et al.*, 1997). The expression of this variant, therefore, would be expected to provide an additional source of interindividual variability in ibuprofen disposition. As yet no specific studies have been performed to detect any change in the proportions of CYP2C9 polymorphism phenotype in cross-sections of the population of different ages.

Age-related differences in elimination via the glucuronidation pathway were only apparent for flurbiprofen, with the formation clearances for glucuronidation of both enantiomers of the drug being lower by 60% in the elderly. The lack of any similar age-associated alterations in the glucuronidation of ibuprofen would suggest that the reaction is mediated by different isoenzymes of the UDP-glucuronosyltransferase for the different drugs.

Interestingly, the extent of (*R*)-ibuprofen undergoing metabolic inversion to the active *S*-enantiomer did not show age-related differences, with the young and elderly having F_{inv} values of 0.68 and 0.66 respectively (Chapter 3). It is therefore likely that expression of hepatic long chain fatty acid CoA synthetase, which modulates the rate-limiting step of the inversion process, is not altered significantly with advancing age.

Overall, it appears that change in the disposition of ibuprofen with age stems from a combination of an increase in the unbound fraction and a decrease in the unbound clearance, due to impaired oxidative metabolism, of the *S*-enantiomer. These factors operate in opposite directions such that at steady state the total serum concentrations would be similar between the two groups but unbound concentrations would be higher in the elderly, this is reflected by the elderly having a significantly greater AUC by 25% for unbound (*S*)-ibuprofen then the young (Chapter 3). Age-related differences in the disposition of flurbiprofen, on the other hand, seem to be primarily due to reduced clearance as a result of a general decline in hepatic metabolising capacity. Consequently, the elderly have greater exposure to both enantiomers of the drug with enantiomeric AUCs typically 60 % larger in this age group; parallel changes in unbound AUCs would be expected since age does not appear to have a major influence on the plasma protein binding of flurbiprofen. As it is the unbound concentrations of these drugs that are important for their pharmacodynamic effects, the elderly are therefore potentially exposed to increased effect and risk of toxicity at normal therapeutic doses.

A variety of physiological and morphological changes occur in the ageing liver which may influence hepatic drug elimination. These include a decrease in size, as a proportion of body weight, from about 2.5% of bodyweight at age 50 to 1.6% of bodyweight at age 90; and a possible fall in blood flow from 1400 ml/min at age 30 to 800 ml/min at age 75 (Woodhouse and Wynne, 1987). However, the effect of age on the drug metabolising capability of the liver *per se* is not clear with limited data available in the literature. There is "normally" considerable variation between healthy individuals in hepatic metabolism due to genetic factors, caffeine and cigarette consumption and nutritional status as well as age.

Recently, the effect of age has been investigated on CYP-mediated metabolism in 226 subjects with "equal" histopathological conditions (Sotaniemi *et al.*, 1997). The subjects were classified by age in decades and the CYP contents of liver biopsy samples and the rate of antipyrine elimination from plasma assessed simultaneously. It appears that age has a reducing effect on oxidative drug metabolism in humans with CYP content (an *in vitro* parameter) altered stepwise, whereas antipyrine metabolism (an *in vivo* index) declined linearly after 40 years (Sotaniemi *et al.*, 1997). It is worth noting that other factors may also contribute to age related alterations in the rate of oxidative metabolism, which include the affinity of the relevant CYP450s for the drug, the

concentrations of CYP reductase and the integrity of the lipid domain (Durnas *et al.*, 1990).

It has been suggested that the effect of ageing on phase II conjugation reactions appears to be less pronounced. Several authors have examined the effect of age upon *in vitro* UDP-glucuronyltransferase activities and have reported no change in Fischer 344 rates or minimal changes only (10-20%) in Wistar rats (Woodhouse and James, 1990). Similarly sulphation of paracetamol in isolated hepatocytes was reported to be slightly decreased in old rats but not in old mice, when compared to young adults (Woodhouse and James, 1990). No data are available on the effects of age on these enzymes in human liver.

7.4.3 Toxicological implications

The changes in pharmacodynamic response with the altered pharmacokinetics of (*S*)-ibuprofen in the elderly were assessed by monitoring serum thromboxane B_2 concentrations (TXB_2) as an indicator of cyclooxygenase-1 (COX-1) activity. COX-1 is constitutively expressed in many tissues including the gastrointestinal tract were it is responsible for the generation of cytoprotective prostaglandins (Vane *et al.*, 1998). These prostaglandins provide protection to the gastrointestinal mucosa by reducing gastric acid production, increasing bicarbonate ion and mucus production, and maintaining blood flow to the intestinal mucosa (Soll, 1991). The pharmacodynamic investigations performed, indicate that the time-course of inhibitory activity on TXB_2 generation is in close correlation with serum unbound concentrations of (*S*)-ibuprofen (Chapter 5). The transient nature of the activity is reflective of the drug's ability to cause rapid reversible competitive inhibition of COX (Wu, 1998). Comparison of the TXB_2 profiles between the two age groups mirror the differences in the unbound (*S*)-ibuprofen serum profile and thus indicate that inhibitory activity has a more rapid onset and is longer-lived in the elderly in comparison to the young. The presence of significant residual activity after 24 hours post-dose in the elderly suggests that age-related changes in response may not be exclusively dose related but also due to attenuated sensitivity as a result of idiosyncratic pharmacodynamic alterations. For example, changes in enzyme number and affinity or blunted homeostatic mechanisms in the elderly could be possible associated factors (Hammerlein *et al.*, 1998). The above findings suggest that altered disposition of ibuprofen in the elderly does increase the risk

of adverse reactions due to the strong relationship between activity and unbound serum concentration of (*S*)-ibuprofen.

Although pharmacodynamic studies were not performed for flurbiprofen, the likelihood of toxicity in the elderly is more apparent than with ibuprofen since flurbiprofen enantiomers have comparatively longer elimination half-lives and age related alterations in disposition were more profound. This is also likely to be intensified by the fact that (*S*)-flurbiprofen has a more potent activity on COX; since in contrast to ibuprofen, flurbiprofen causes slowly-reversible time-dependent inactivation (Wu, 1998). After the initial competitive inhibition of COX, flurbiprofen is believed to cause active site structural changes to facilitate slowly reversible inhibition; this phase of inhibition takes minutes to accomplish and, hence, is time-dependent. Furthermore, the increased exposure of the elderly to the *R*-enantiomer, which is relatively inactive in terms of COX inhibition, can not be assumed to be of little consequence. Since after long term administration of either the racemate or the individual enantiomers of flurbiprofen to rats, Wechter *et al.* (1993) found that the *R*-enantiomer alone did not elicit gastrointestinal lesions, but the racemate proved to be 2 to 4 fold more ulcerogenic in enantiomerically equivalent doses than the *S*-enantiomer. Recently, it has been shown that the *R*-enantiomer might exacerbate gastrointestinal toxicity in the rat by causing uncoupling of mitochondrial oxidative phosphorylation which consequently lead to increased intestinal permeability (Mahmud *et al.*, 1998). Thus taking all these factors into consideration, it would seem that flurbiprofen should be used with considerable caution in the elderly population.

It is also important to note that in addition to altered pharmacokinetics, increasing age is associated with many alterations in gastrointestinal physiology that may contribute to an increased risk of NSAID-induced gastrointestinal complications. Free radical scavenging functions are decreased, vascular integrity is diminished, and perhaps most important prostaglandin biochemistry is significantly altered (Solomon and Gurwitz, 1997). Studies in animals (Lee and Feldman, 1994) and humans have demonstrated reduced gastrointestinal prostaglandin levels with age. Goto *et al.* (1992) sampled the gastric mucosa in healthy volunteers for the concentration of four prostaglandins: 6-keto-prostaglandin $F_{1\alpha}$, prostaglandin $F_{2\alpha}$, prostaglandin E_2 and prostaglandin D_2. The concentrations found in individuals over 70 years of age were half those of subjects less than 70. Cryer *et al.* (1992) also sampled the gastric mucosa from

healthy volunteers and found lower prostaglandin $F_{2\alpha}$, and prostaglandin E_2 levels in volunteers aged 52 to 72 compared with younger subjects aged between 21 to 40.

Ageing occurs at different rates, which may be influenced by genetic predisposition, e.g. type II diabetes, or by environmental and lifestyle factors; therefore the elderly can be viewed as a very diverse population group. In addition the ageing process is often superimposed by various disease conditions to which the elderly are more often susceptible e.g. renal dysfunction and musculoskeletal disorders. As a result of these multiple pathologies, the elderly are generally prescribed more drugs than the young, potentially leading to an increased risk of NSAID-related adverse reactions due to drug-disease and drug-drug interactions. Of primary importance, with regards to disposition of the NSAIDs, is if these interactions effect protein binding and/or metabolism, including the chiral inversion process. Thus the findings of this study should be considered with these factors also in mind.

7.4.4 Concluding Remarks

The results of the present study indicate age-related alterations in the disposition of both 2-arylpropionic acid drugs; such that the elderly have greater exposure to the unbound *S*-enantiomer of ibuprofen and both enantiomers in the case of flurbiprofen. These results taken collectively with the greater susceptibility to adverse reactions and the increased prevalence of certain comorbid conditions and concomitant drug therapies, suggest that the elderly are particularly at risk from the gastrointestinal complications of these well-established and widely-used NSAIDs. It is also obvious that many more studies investigating the age-related alterations in pharmacodynamic activity in combination with the enantiomeric pharmacokinetics of NSAIDs are necessary, to better appreciate the clinical implications of such changes and thus increase the safe and efficacious use of these drugs in the ever increasing elderly population.

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APPENDIX

Appendix 1: Demographic details and serum albumin concentrations for the young and elderly volunteers of the ibuprofen study.

Ibuprofen study: Young					
Subject Code	**Sex**	**Age (years)**	**Weight (kg)**	**Height (cm)**	**Albumin (g/L)**
Iy1	male	31	78.0	177	49.0
Iy2	male	22	77.0	182	50.1
Iy3	female	25	63.5	168	52.8
Iy4	female	23	75.0	172	50.3
Iy5	female	28	64.0	170	49.2
Iy6	female	23	77.0	180	56.5
Iy7	male	20	84.0	187	50.6
Iy8	male	21	67.0	177	51.0
Mean	-	24.1	73.2	176.6	51.2
s.d.	-	3.7	7.5	6.4	2.4
Ibuprofen study: Elderly					
Ie1	male	83	83.5	179	50.9
Ie2	male	84	75.5	166	47.9
Ie3	male	71	63.0	174	49.4
Ie4	male	72	73.0	172	53.3
Ie5	male	66	83.0	175	47.5
Ie6	male	71	89.0	175	52.9
Ie7	male	83	55.0	177	46.1
Ie8	female	75	58.0	157	52.2
Mean	-	75.6	72.5	171.9	50.0
s.d.	-	6.8	12.6	7.1	2.7

Comparison of the means for weight, height and serum albumin concentration between the young and elderly groups using a T-test for independent samples, revealed no significant age-related differences.

Appendix 2: Serum concentrations (mcg/ml) for (*R*)- and (*S*)-ibuprofen in individual healthy young volunteers following the oral administration of 400 mg racemic ibuprofen.

	(*R*)-ibuprofen (mcg/ml)									
Time (hr)	Iy1	Iy2	Iy3	Iy4	Iy5	Iy6	Iy7	Iy8	mean	s.d.
0.25	7.94	0.00	0.17	1.43	0.47	0.11	0.00	0.00	1.27	2.74
0.50	13.05	0.00	2.73	1.82	2.59	0.38	0.45	0.14	2.65	4.35
0.75	13.99	0.13	11.43	5.58	4.84	0.91	2.55	0.33	4.97	5.22
1.00	16.13	0.19	14.02	10.00	7.49	1.55	3.40	0.52	6.66	6.23
1.50	15.46	1.98	14.88	14.09	8.48	2.04	4.20	2.28	7.93	6.08
2.00	14.49	3.78	13.33	15.78	17.23	2.12	4.06	13.43	10.53	6.12
2.50	11.62	10.79	12.90	14.91	22.96	2.93	11.63	17.62	13.17	5.78
3.00	11.16	15.21	12.68	14.26	19.38	3.00	11.02	17.42	13.02	4.99
3.50	9.11	14.96	10.52	12.79	17.42	2.95	9.98	14.11	11.48	4.43
4.00	5.39	14.58	7.26	11.69	12.78	3.67	8.70	11.54	9.45	3.82
6.00	4.25	6.28	1.62	4.50	5.79	11.32	2.67	3.39	4.98	2.99
8.00	1.63	3.59	0.50	2.30	1.91	3.48	1.40	0.92	1.97	1.12
10.00	0.78	1.86	0.35	1.36	0.62	1.22	0.46	0.47	0.89	0.54
24.00	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.01	0.03

	(*S*)-ibuprofen (mcg/ml)									
Time (hr)	Iy1	Iy2	Iy3	Iy4	Iy5	Iy6	Iy7	Iy8	mean	s.d.
0.25	7.55	0.00	0.11	1.25	0.32	0.06	0.00	0.00	1.16	2.62
0.50	13.63	0.00	2.72	1.76	2.01	0.28	0.48	0.13	2.63	4.56
0.75	14.22	0.12	11.37	5.32	3.85	0.73	2.71	0.33	4.83	5.29
1.00	16.78	0.31	14.02	9.32	6.04	1.27	3.93	0.57	6.53	6.30
1.50	15.79	2.16	16.15	14.32	6.80	1.59	5.08	1.77	7.96	6.45
2.00	14.55	4.00	16.23	14.93	13.19	1.74	5.21	11.64	10.19	5.65
2.50	11.48	11.97	15.69	14.90	16.84	2.29	13.20	13.99	12.55	4.52
3.00	10.81	15.88	15.16	13.55	14.35	2.31	13.65	14.12	12.48	4.37
3.50	9.48	16.10	13.35	11.43	13.90	2.32	13.70	12.38	11.58	4.21
4.00	5.47	15.45	12.12	11.00	10.44	2.95	12.51	9.50	9.93	4.00
6.00	5.05	7.41	7.99	7.58	7.91	16.14	8.01	6.77	8.36	3.29
8.00	2.29	5.15	4.20	3.38	4.01	11.83	3.70	3.45	4.75	2.97
10.00	1.08	1.89	2.60	1.95	1.90	7.44	2.07	1.49	2.55	2.02
24.00	0.00	0.00	0.00	0.00	0.00	0.34	0.00	0.00	0.04	0.12

Appendix 3: Serum concentrations (mcg/ml) for (*R*)- and (*S*)-ibuprofen in individual healthy elderly volunteers following the oral administration of 400 mg racemic ibuprofen.

	(*R*)-ibuprofen (mcg/ml)									
Time (hr)	**Ie1**	**Ie2**	**Ie3**	**Ie4**	**Ie5**	**Ie6**	**Ie7**	**Ie8**	**mean**	**s.d.**
0.25	1.45	3.62	1.20	2.05	2.57	0.46	4.02	10.71	3.26	3.24
0.50	8.28	6.63	10.33	9.72	5.36	0.73	11.86	15.33	8.53	4.42
0.75	12.00	8.75	12.96	11.38	9.20	1.37	15.35	18.36	11.17	5.06
1.00	17.73	14.47	14.44	13.51	9.69	4.96	16.36	20.09	13.91	4.74
1.50	17.85	14.80	13.82	14.57	11.46	9.03	17.18	21.44	15.02	3.86
2.00	19.25	14.67	13.67	15.47	11.01	11.28	20.38	21.44	15.90	4.03
2.50	18.40	13.39	12.80	15.42	11.27	11.99	16.39	20.19	14.98	3.18
3.00	17.73	13.07	12.48	14.30	10.16	10.15	14.04	17.03	13.62	2.80
3.50	13.90	12.04	12.03	13.83	9.89	9.24	12.17	16.65	12.47	2.36
4.00	11.11	11.43	11.22	11.35	7.65	7.67	11.54	15.57	10.94	2.50
6.00	4.35	5.43	4.40	3.32	2.29	3.09	3.41	6.74	4.13	1.43
8.00	3.05	2.45	1.93	0.95	1.53	1.67	1.13	2.56	1.91	0.73
10.00	2.33	1.03	1.17	0.38	0.77	0.67	0.21	1.29	0.98	0.66
24.00	0.10	0.19	0.07	0.00	0.00	0.00	0.00	0.00	0.05	0.07

	(*S*)-ibuprofen (mcg/ml)									
Time (hr)	**Ie1**	**Ie2**	**Ie3**	**Ie4**	**Ie5**	**Ie6**	**Ie7**	**Ie8**	**mean**	**s.d.**
0.25	1.25	3.21	1.03	1.77	2.50	0.31	2.60	8.40	2.63	2.51
0.50	7.97	5.61	9.65	8.83	4.95	0.64	7.78	11.68	7.14	3.39
0.75	10.41	7.68	13.44	10.36	8.55	1.38	10.53	13.48	9.48	3.86
1.00	13.08	12.11	14.70	12.52	8.73	3.67	11.33	14.52	11.33	3.63
1.50	16.48	13.44	14.17	13.23	11.38	7.48	11.77	15.58	12.94	2.80
2.00	14.44	14.01	13.91	14.10	10.11	8.77	13.19	15.65	13.02	2.34
2.50	13.62	12.81	12.90	12.58	9.29	10.33	10.49	14.30	12.04	1.78
3.00	12.78	11.58	12.60	13.10	9.07	9.33	9.39	12.43	11.29	1.73
3.50	10.03	11.25	10.89	12.19	7.21	8.55	8.81	11.92	10.11	1.77
4.00	9.17	9.51	10.37	11.17	5.82	7.36	7.95	10.88	9.03	1.86
6.00	5.71	6.77	6.25	8.38	2.66	4.61	5.75	6.79	5.87	1.69
8.00	4.81	5.33	3.36	4.63	2.44	3.27	3.18	4.75	3.97	1.03
10.00	3.02	3.52	2.43	2.73	1.96	1.29	1.77	2.62	2.42	0.72
24.00	0.23	0.32	0.17	0.00	0.10	0.00	0.00	0.00	0.10	0.13

Appendix 4: Cumulative urinary excretion of ibuprofen enantiomers in individual healthy young volunteers following the oral administration of 400 mg racemic ibuprofen.

	total ibuprofen (% of administered dose)											
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Iy1	0.02	0.23	0.04	0.38	0.17	1.66	0.29	2.79	0.40	3.88	1.11	10.73
Iy2	0.06	0.63	0.09	0.91	0.13	1.26	0.20	1.92	0.27	2.66	0.89	8.75
Iy3	0.03	0.47	0.10	1.38	0.15	2.07	0.23	3.16	0.36	4.95	1.01	14.04
Iy4	0.03	0.28	0.05	0.55	0.09	0.92	0.15	1.59	0.15	1.65	1.09	11.68
Iy5	0.02	0.28	0.03	0.51	0.08	1.23	0.16	2.42	0.31	4.75	0.82	12.58
Iy6	0.18	2.27	0.26	3.31	0.29	3.70	0.33	4.26	0.42	5.40	0.87	11.20
Iy7	0.14	1.63	0.22	2.51	0.32	3.63	0.35	3.95	0.39	4.43	0.67	7.63
Iy8	0.05	0.61	0.10	1.14	0.15	1.71	0.28	3.16	0.49	5.64	1.13	12.98
mean	0.07	0.80	0.11	1.34	0.17	2.02	0.25	2.91	0.35	4.17	0.95	11.20
s.d.	0.06	0.75	0.08	1.05	0.09	1.07	0.08	0.93	0.10	1.38	0.16	2.15

	conjugated ibuprofen (% of administered dose)											
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Iy1	0.02	0.22	0.04	0.36	0.16	1.58	0.28	2.65	0.39	3.69	1.07	10.19
Iy2	0.05	0.57	0.08	0.82	0.12	1.14	0.18	1.74	0.25	2.41	0.81	7.92
Iy3	0.03	0.45	0.10	1.33	0.15	1.99	0.23	3.04	0.35	4.76	0.99	13.49
Iy4	0.03	0.25	0.05	0.49	0.09	0.82	0.14	1.41	0.14	1.47	1.02	10.39
Iy5	0.02	0.26	0.03	0.47	0.08	1.12	0.15	2.21	0.29	4.33	0.78	11.48
Iy6	0.17	2.14	0.25	3.12	0.28	3.49	0.32	4.02	0.41	5.09	0.84	10.57
Iy7	0.13	1.45	0.20	2.24	0.29	3.23	0.32	3.52	0.35	3.95	0.61	6.79
Iy8	0.04	0.52	0.09	0.97	0.13	1.46	0.25	2.70	0.44	4.81	1.00	11.08
mean	0.06	0.73	0.10	1.22	0.16	1.85	0.23	2.66	0.33	3.81	0.89	10.24
s.d.	0.06	0.69	0.08	0.98	0.08	1.00	0.07	0.87	0.10	1.27	0.16	2.08

	free ibuprofen (% of administered dose)											
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Iy1	0.00	0.01	0.00	0.02	0.01	0.08	0.01	0.14	0.02	0.20	0.04	0.54
Iy2	0.01	0.06	0.01	0.09	0.01	0.12	0.02	0.18	0.02	0.25	0.08	0.83
Iy3	0.00	0.02	0.00	0.05	0.00	0.08	0.00	0.12	0.01	0.19	0.02	0.55
Iy4	0.00	0.03	0.00	0.06	0.01	0.10	0.01	0.18	0.01	0.18	0.07	1.29
Iy5	0.00	0.03	0.00	0.04	0.00	0.11	0.01	0.21	0.02	0.42	0.05	1.10
Iy6	0.01	0.13	0.01	0.19	0.01	0.21	0.01	0.24	0.01	0.31	0.03	0.63
Iy7	0.01	0.18	0.02	0.28	0.03	0.40	0.03	0.43	0.04	0.49	0.07	0.84
Iy8	0.01	0.09	0.01	0.17	0.02	0.25	0.03	0.46	0.06	0.83	0.13	1.90
mean	0.00	0.07	0.01	0.11	0.01	0.17	0.02	0.25	0.02	0.36	0.06	0.96
s.d.	0.00	0.06	0.01	0.09	0.01	0.11	0.01	0.13	0.02	0.22	0.03	0.46

Appendix 5: Cumulative urinary excretion of hydroxyibuprofen enantiomers in individual healthy young volunteers following the oral administration of 400 mg racemic ibuprofen.

	total hydroxyibuprofen (% of administered dose)											
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Iy1	0.10	0.37	0.16	0.60	0.67	2.60	1.13	4.37	1.58	6.08	4.36	16.80
Iy2	0.17	1.27	0.25	1.63	0.35	2.27	0.53	3.46	0.73	4.79	2.40	15.77
Iy3	0.08	0.62	0.24	1.80	0.35	2.69	0.54	4.12	0.85	6.44	2.40	18.29
Iy4	0.06	0.41	0.13	0.83	0.21	1.37	0.37	2.37	0.38	2.45	2.72	17.40
Iy5	0.10	0.44	0.18	0.80	0.44	1.93	0.86	3.81	1.69	7.49	4.47	19.84
Iy6	0.61	4.26	0.89	6.20	0.99	6.93	1.14	7.98	1.45	10.13	3.00	20.99
Iy7	0.73	3.98	1.13	6.14	1.63	8.86	1.77	9.64	1.98	10.81	3.42	18.64
Iy8	0.18	0.73	0.33	1.37	0.49	2.06	0.91	3.82	1.63	6.81	3.74	15.65
mean	0.25	1.51	0.41	2.42	0.64	3.59	0.91	4.95	1.29	6.88	3.31	17.92
s.d.	0.26	1.64	0.38	2.35	0.46	2.74	0.45	2.50	0.56	2.71	0.82	1.89

	conjugated hydroxyibuprofen (% of administered dose)											
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Iy1	0.02	0.22	0.03	0.36	0.14	1.58	0.24	2.65	0.34	3.69	0.93	10.20
Iy2	0.07	0.79	0.10	1.14	0.14	1.58	0.21	2.41	0.29	3.34	0.95	10.99
Iy3	0.02	0.41	0.06	1.18	0.08	1.77	0.13	2.71	0.20	4.24	0.56	12.04
Iy4	0.01	0.25	0.02	0.51	0.03	0.84	0.06	1.46	0.06	1.51	0.45	10.68
Iy5	0.02	0.23	0.04	0.42	0.09	1.00	0.18	1.98	0.35	3.89	0.94	10.29
Iy6	0.08	1.94	0.12	2.82	0.13	3.15	0.15	3.63	0.19	4.61	0.40	9.55
Iy7	0.12	2.17	0.18	3.35	0.26	4.84	0.29	5.26	0.32	5.90	0.55	10.18
Iy8	0.01	0.32	0.02	0.61	0.03	0.91	0.05	1.69	0.09	3.01	0.21	6.92
mean	0.04	0.79	0.07	1.30	0.11	1.96	0.16	2.72	0.23	3.77	0.62	10.11
s.d.	0.04	0.80	0.06	1.15	0.07	1.38	0.08	1.23	0.11	1.28	0.28	1.48

	free hydroxyibuprofen (% of administered dose)											
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Iy1	0.08	0.14	0.12	0.24	0.53	1.02	0.89	1.72	1.24	2.39	3.43	6.60
Iy2	0.10	0.49	0.15	0.50	0.21	0.69	0.32	1.05	0.44	1.45	1.44	4.78
Iy3	0.06	0.21	0.18	0.61	0.27	0.92	0.42	1.41	0.65	2.20	1.84	6.25
Iy4	0.05	0.16	0.11	0.32	0.18	0.53	0.31	0.92	0.32	0.95	2.27	6.72
Iy5	0.08	0.21	0.14	0.39	0.34	0.93	0.68	1.84	1.34	3.61	3.54	9.55
Iy6	0.53	2.32	0.77	3.38	0.86	3.78	0.99	4.35	1.25	5.52	2.60	11.45
Iy7	0.61	1.81	0.94	2.79	1.36	4.02	1.48	4.37	1.66	4.91	2.87	8.46
Iy8	0.17	0.41	0.31	0.76	0.47	1.15	0.86	2.13	1.54	3.80	3.53	8.73
mean	0.21	0.72	0.34	1.12	0.53	1.63	0.74	2.22	1.05	3.10	2.69	7.82
s.d.	0.23	0.85	0.33	1.23	0.40	1.42	0.40	1.38	0.51	1.63	0.80	2.13

Appendix 6: Cumulative urinary excretion of carboxyibuprofen stereoisomers in individual healthy young volunteers following the oral administration of 400 mg racemic ibuprofen.

		total carboxyibuprofen (% of administered dose)											
		0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject		*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Iy1	*2'R-*	0.11	0.28	0.18	0.46	0.78	1.98	1.31	3.34	1.83	4.64	5.05	12.82
	2'S-	0.06	0.33	0.10	0.54	0.42	2.36	0.71	3.97	0.99	5.52	2.72	15.26
Iy2	*2'R-*	0.33	0.91	0.48	1.32	0.67	1.84	1.02	2.80	1.42	3.87	4.66	12.74
	2'S-	0.23	1.23	0.34	1.78	0.47	2.48	0.72	3.78	0.99	5.23	3.26	17.21
Iy3	*2'R-*	0.13	0.45	0.38	1.33	0.57	1.98	0.87	3.03	1.36	4.74	3.87	13.47
	2'S-	0.07	0.54	0.21	1.57	0.31	2.35	0.48	3.60	0.75	5.63	2.13	15.99
Iy4	*2'R-*	0.10	0.29	0.21	0.59	0.34	0.97	0.59	1.69	0.61	1.74	4.32	12.37
	2'S-	0.06	0.36	0.11	0.72	0.19	1.19	0.32	2.07	0.33	2.14	2.36	15.18
Iy5	*2'R-*	0.13	0.42	0.24	0.75	0.58	1.82	1.15	3.58	2.25	7.04	5.97	18.65
	2'S-	0.09	0.48	0.16	0.87	0.38	2.10	0.74	4.15	1.46	8.16	3.86	21.61
Iy6	*2'R-*	0.89	3.50	1.30	5.10	1.45	5.71	1.67	6.57	2.12	8.33	4.39	17.27
	2'S-	0.58	4.59	0.85	6.69	0.95	7.48	1.09	8.62	1.39	10.93	2.88	22.66
Iy7	*2'R-*	1.02	3.32	1.57	5.12	2.27	7.38	2.47	8.03	2.77	9.01	4.77	15.53
	2'S-	0.54	3.76	0.83	5.80	1.19	8.36	1.30	9.09	1.45	10.21	2.47	17.59
Iy8	*2'R-*	0.18	0.53	0.33	0.99	0.50	1.49	0.92	2.76	1.65	4.92	3.78	11.31
	2'S-	0.14	0.73	0.27	1.37	0.40	2.07	0.74	3.82	1.32	6.82	3.04	15.67
mean	*2'R-*	0.36	1.21	0.59	1.96	0.90	2.90	1.25	3.98	1.75	5.54	4.60	14.27
	2'S-	0.22	1.50	0.36	2.42	0.54	3.55	0.76	4.89	1.09	6.83	2.84	17.65
s.d	*2'R-*	0.38	1.37	0.54	1.97	0.65	2.32	0.59	2.16	0.66	2.42	0.70	2.60
	2'S-	0.22	1.69	0.31	2.41	0.34	2.74	0.31	2.53	0.40	2.87	0.55	2.91

Appendix 6: continued

		conjugated carboxyibuprofen (% of administered dose)											
		0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject		*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Iy1	*2'R-*	0.04	0.10	0.06	0.17	0.26	0.70	0.43	1.19	0.61	1.65	1.68	4.56
	2'S-	0.01	0.12	0.02	0.20	0.09	0.87	0.16	1.46	0.23	2.03	0.62	5.60
Iy2	*2'R-*	0.12	0.30	0.18	0.44	0.25	0.61	0.39	0.93	0.54	1.29	1.77	4.25
	2'S-	0.05	0.44	0.08	0.64	0.11	0.89	0.17	1.36	0.23	1.88	0.77	6.1[illegible]
Iy3	*2'R-*	0.03	0.11	0.10	0.35	0.15	0.52	0.23	0.79	0.35	1.24	1.01	3.52
	2'S-	0.01	0.16	0.03	0.47	0.05	0.70	0.08	1.08	0.12	1.69	0.35	4.80
Iy4	*2'R-*	0.02	0.07	0.06	0.15	0.09	0.25	0.16	0.44	0.16	0.44	1.15	3.1[illegible]
	2'S-	0.01	0.10	0.02	0.20	0.03	0.33	0.05	0.57	0.05	0.59	0.38	4.21
Iy5	*2'R-*	0.04	0.16	0.08	0.28	0.20	0.69	0.41	1.34	0.79	2.64	2.11	7.01
	2'S-	0.03	0.18	0.05	0.33	0.11	0.80	0.21	1.58	0.42	3.11	1.10	8.23
Iy6	*2'R-*	0.16	0.35	0.24	0.51	0.27	0.57	0.31	0.66	0.39	0.83	0.81	1.72
	2'S-	0.06	0.92	0.10	1.34	0.11	1.50	0.12	1.74	0.16	2.20	0.34	4.56
Iy7	*2'R-*	0.19	0.71	0.29	1.09	0.42	1.57	0.46	1.71	0.51	1.92	0.88	3.31
	2'S-	0.07	0.81	0.10	1.25	0.14	1.79	0.15	1.94	0.16	2.19	0.29	3.77
Iy8	*2'R-*	0.01	0.02	0.02	0.05	0.03	0.07	0.05	0.13	0.09	0.22	0.20	0.52
	2'S-	0.01	0.04	0.02	0.08	0.02	0.13	0.05	0.23	0.08	0.41	0.20	0.95
mean	*2'R-*	0.08	0.23	0.13	0.38	0.21	0.62	0.31	0.90	0.43	1.28	1.20	3.51
	2'S-	0.03	0.35	0.05	0.56	0.08	0.88	0.12	1.25	0.18	1.76	0.51	4.79
s.d	*2'R-*	0.07	0.23	0.10	0.33	0.12	0.44	0.15	0.51	0.23	0.80	0.62	1.93
	2'S-	0.03	0.34	0.04	0.48	0.04	0.55	0.06	0.59	0.12	0.89	0.30	2.09

		free carboxyibuprofen (% of administered dose)											
		0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject		*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Iy1	*2'R-*	0.07	0.18	0.12	0.29	0.52	1.28	0.88	2.15	1.22	2.99	3.37	8.26
	2'S-	0.05	0.21	0.08	0.34	0.33	1.49	0.55	2.51	0.76	3.49	2.10	9.66
Iy2	*2'R-*	0.21	0.61	0.30	0.88	0.42	1.23	0.63	1.87	0.88	2.58	2.89	8.49
	2'S-	0.18	0.79	0.26	1.14	0.36	1.59	0.55	2.42	0.76	3.35	2.49	11.04
Iy3	*2'R-*	0.10	0.34	0.28	0.98	0.42	1.46	0.64	2.24	1.01	3.50	2.86	9.95
	2'S-	0.06	0.38	0.18	1.10	0.26	1.65	0.40	2.52	0.63	3.94	1.78	11.19
Iy4	*2'R-*	0.08	0.22	0.15	0.44	0.25	0.72	0.43	1.25	0.45	1.30	3.17	9.20
	2'S-	0.05	0.26	0.09	0.52	0.16	0.86	0.27	1.50	0.28	1.55	1.98	10.97
Iy5	*2'R-*	0.09	0.26	0.16	0.47	0.38	1.13	0.74	2.24	1.46	4.40	3.86	11.64
	2'S-	0.06	0.30	0.11	0.54	0.27	1.30	0.53	2.57	1.04	5.05	2.76	13.38
Iy6	*2'R-*	0.73	3.15	1.06	4.59	1.18	5.14	1.36	5.91	1.73	7.50	3.58	15.55
	2'S-	0.52	3.67	0.75	5.35	0.84	5.98	0.97	6.88	1.23	8.73	2.54	18.10
Iy7	*2'R-*	0.83	2.61	1.28	4.03	1.85	5.81	2.01	6.32	2.26	7.09	3.89	12.22
	2'S-	0.47	2.95	0.73	4.55	1.05	6.57	1.15	7.15	1.29	8.02	2.22	13.82
Iy8	*2'R-*	0.17	0.51	0.31	0.94	0.47	1.42	0.87	2.63	1.56	4.70	3.58	10.79
	2'S-	0.13	0.69	0.25	1.29	0.38	1.94	0.69	3.59	1.24	6.41	2.84	14.72
mean	*2'R-*	0.29	0.99	0.46	1.58	0.69	2.27	0.95	3.08	1.32	4.26	3.40	10.76
	2'S-	0.19	1.16	0.31	1.85	0.46	2.67	0.64	3.64	0.90	5.07	2.34	12.86
s.d	*2'R-*	0.31	1.19	0.45	1.71	0.55	2.00	0.51	1.92	0.56	2.15	0.40	2.40
	2'S-	0.19	1.36	0.28	1.95	0.31	2.25	0.29	2.16	0.36	2.48	0.38	2.73

Appendix 7: Cumulative urinary excretion of ibuprofen enantiomers in individual healthy elderly volunteers following the oral administration of 400 mg racemic ibuprofen.

	total ibuprofen (% of administered dose)											
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Ie1	0.07	1.08	0.12	1.74	0.12	1.74	0.17	2.51	0.20	2.94	0.75	11.07
Ie2	0.24	2.78	0.37	4.25	0.42	4.86	0.51	5.87	0.57	6.61	1.13	13.02
Ie3	0.03	0.33	0.06	0.58	0.10	1.06	0.15	1.59	0.23	2.45	0.79	8.37
Ie4	0.03	0.44	0.06	0.82	0.09	1.24	0.14	1.87	0.35	4.75	1.38	18.74
Ie5	0.11	1.08	0.21	1.95	0.30	2.84	0.39	3.72	0.51	4.84	1.50	14.20
Ie6	0.04	0.47	0.09	0.96	0.11	1.20	0.13	1.40	0.17	1.87	0.63	6.77
Ie7	0.07	0.66	0.16	1.49	0.21	1.98	0.27	2.48	0.32	2.94	0.96	8.93
Ie8	0.03	0.28	0.07	0.56	0.09	0.76	0.16	1.32	0.23	1.94	1.16	9.58
mean	0.08	0.89	0.14	1.54	0.18	1.96	0.24	2.60	0.32	3.54	1.04	11.34
s.d.	0.07	0.82	0.11	1.21	0.12	1.34	0.14	1.54	0.15	1.68	0.31	3.86

	conjugated ibuprofen (% of administered dose)											
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Ie1	0.06	1.02	0.11	1.65	0.11	1.65	0.16	2.38	0.18	2.79	0.69	10.50
Ie2	0.23	2.66	0.35	4.06	0.40	4.64	0.48	5.61	0.54	6.31	1.06	12.44
Ie3	0.03	0.31	0.06	0.55	0.09	1.00	0.14	1.50	0.22	2.31	0.74	7.90
Ie4	0.03	0.38	0.05	0.71	0.08	1.07	0.12	1.62	0.31	4.10	1.22	16.19
Ie5	0.10	1.01	0.20	1.82	0.28	2.65	0.36	3.47	0.47	4.52	1.39	13.26
Ie6	0.04	0.43	0.08	0.88	0.10	1.09	0.12	1.28	0.15	1.70	0.57	6.17
Ie7	0.06	0.55	0.14	1.24	0.18	1.65	0.23	2.07	0.28	2.45	0.83	7.45
Ie8	0.03	0.25	0.06	0.51	0.08	0.69	0.15	1.19	0.21	1.76	1.06	8.67
mean	0.07	0.83	0.13	1.43	0.16	1.81	0.22	2.39	0.29	3.24	0.94	10.32
s.d.	0.07	0.80	0.10	1.17	0.12	1.30	0.13	1.50	0.14	1.61	0.28	3.42

	free ibuprofen (% of administered dose)											
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Ie1	0.01	0.06	0.01	0.09	0.01	0.09	0.02	0.13	0.02	0.15	0.07	0.57
Ie2	0.01	0.13	0.02	0.19	0.03	0.22	0.03	0.26	0.03	0.30	0.07	0.58
Ie3	0.00	0.02	0.00	0.03	0.01	0.06	0.01	0.09	0.02	0.14	0.05	0.47
Ie4	0.00	0.06	0.01	0.11	0.01	0.17	0.02	0.25	0.04	0.65	0.16	2.55
Ie5	0.01	0.07	0.02	0.13	0.02	0.19	0.03	0.25	0.04	0.32	0.11	0.94
Ie6	0.00	0.04	0.01	0.09	0.01	0.11	0.01	0.13	0.02	0.17	0.06	0.60
Ie7	0.01	0.11	0.02	0.25	0.03	0.33	0.04	0.41	0.04	0.49	0.13	1.48
Ie8	0.00	0.03	0.01	0.05	0.01	0.07	0.01	0.13	0.02	0.19	0.10	0.91
mean	0.01	0.06	0.01	0.12	0.02	0.15	0.02	0.21	0.03	0.30	0.09	1.02
s.d.	0.00	0.04	0.01	0.07	0.01	0.09	0.01	0.11	0.01	0.18	0.04	0.70

Appendix 8: Cumulative urinary excretion of hydroxyibuprofen enantiomers in individual healthy elderly volunteers following the oral administration of 400 mg racemic ibuprofen.

	total hydroxyibuprofen (% of administered dose)											
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject	***R***	***S***	***R***	***S***	***R***	***S***	***R***	***S***	***R***	***S***	***R***	***S***
Ie1	0.28	1.48	0.46	2.39	0.46	2.39	0.66	3.45	0.77	4.04	2.90	15.24
Ie2	0.37	2.69	0.57	4.11	0.65	4.70	0.78	5.68	0.88	6.39	1.74	12.60
Ie3	0.07	0.37	0.12	0.64	0.21	1.17	0.32	1.77	0.49	2.73	1.68	9.32
Ie4	0.07	0.43	0.13	0.81	0.20	1.23	0.30	1.84	0.77	4.68	3.04	18.51
Ie5	0.26	1.34	0.46	2.43	0.68	3.54	0.88	4.64	1.15	6.03	3.37	17.71
Ie6	0.25	1.36	0.51	2.80	0.63	3.51	0.74	4.09	0.99	5.46	3.58	19.81
Ie7	0.24	1.12	0.53	2.52	0.71	3.35	0.89	4.21	1.05	4.99	3.19	15.14
Ie8	0.13	0.61	0.26	1.22	0.36	1.65	0.62	2.86	0.91	4.21	4.51	20.82
mean	0.21	1.18	0.38	2.12	0.49	2.69	0.65	3.57	0.88	4.82	3.00	16.14
s.d.	0.11	0.75	0.18	1.16	0.21	1.28	0.23	1.36	0.20	1.18	0.94	3.86

	conjugated hydroxyibuprofen (% of administered dose)											
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject	***R***	***S***	***R***	***S***	***R***	***S***	***R***	***S***	***R***	***S***	***R***	***S***
Ie1	0.06	0.95	0.10	1.54	0.10	1.54	0.14	2.22	0.17	2.60	0.62	9.79
Ie2	0.02	1.72	0.03	2.63	0.03	3.01	0.04	3.64	0.04	4.09	0.08	8.06
Ie3	0.02	0.24	0.04	0.42	0.07	0.77	0.11	1.16	0.17	1.79	0.57	6.12
Ie4	0.01	0.22	0.01	0.41	0.02	0.62	0.03	0.93	0.07	2.36	0.26	9.32
Ie5	0.04	0.79	0.07	1.43	0.10	2.09	0.14	2.73	0.18	3.55	0.52	10.43
Ie6	0.04	0.80	0.08	1.65	0.09	2.07	0.11	2.42	0.15	3.23	0.53	11.70
Ie7	0.02	0.49	0.04	1.10	0.06	1.46	0.07	1.84	0.09	2.17	0.27	6.60
Ie8	0.02	0.37	0.04	0.73	0.05	0.99	0.08	1.71	0.12	2.52	0.59	12.46
mean	0.03	0.70	0.05	1.24	0.06	1.57	0.09	2.08	0.12	2.79	0.43	9.31
s.d.	0.02	0.50	0.03	0.74	0.03	0.80	0.04	0.87	0.05	0.77	0.20	2.27

	free hydroxyibuprofen (% of administered dose)											
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject	***R***	***S***	***R***	***S***	***R***	***S***	***R***	***S***	***R***	***S***	***R***	***S***
Ie1	0.22	0.53	0.36	0.85	0.36	0.85	0.52	1.23	0.61	1.44	2.28	5.45
Ie2	0.35	0.97	0.54	1.48	0.62	1.69	0.75	2.05	0.84	2.31	1.66	4.54
Ie3	0.04	0.13	0.08	0.22	0.14	0.40	0.21	0.61	0.33	0.94	1.11	3.20
Ie4	0.07	0.22	0.12	0.40	0.18	0.61	0.28	0.91	0.70	2.33	2.77	9.19
Ie5	0.22	0.55	0.39	1.00	0.57	1.46	0.75	1.91	0.97	2.48	2.86	7.28
Ie6	0.21	0.56	0.43	1.15	0.54	1.44	0.63	1.67	0.84	2.24	3.05	8.11
Ie7	0.22	0.63	0.49	1.42	0.65	1.89	0.81	2.37	0.96	2.81	2.92	8.54
Ie8	0.12	0.25	0.23	0.49	0.31	0.66	0.54	1.15	0.79	1.69	3.92	8.36
mean	0.18	0.48	0.33	0.88	0.42	1.13	0.56	1.49	0.76	2.03	2.57	6.83
s.d.	0.10	0.27	0.17	0.47	0.20	0.56	0.22	0.61	0.21	0.62	0.87	2.17

Appendix 9: Cumulative urinary excretion of carboxyibuprofen stereoisomers in individual healthy elderly volunteers following the oral administration of 400 mg racemic ibuprofen.

		total carboxyibuprofen (% of administered dose)											
		0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject		*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Ie1	*2'R-*	0.52	1.54	0.83	2.48	0.83	2.48	1.20	3.58	1.41	4.20	5.31	15.84
	2'S-	0.38	1.83	0.62	2.94	0.62	2.94	0.89	4.25	1.05	4.98	3.95	18.78
Ie2	*2'R-*	0.56	1.69	0.86	2.58	0.99	2.95	1.19	3.57	1.34	4.01	2.64	7.91
	2'S-	0.45	2.24	0.69	3.43	0.79	3.92	0.95	4.74	1.07	5.33	2.11	10.50
Ie3	*2'R-*	0.12	0.36	0.20	0.62	0.37	1.14	0.56	1.71	0.86	2.64	2.92	9.01
	2'S-	0.09	0.46	0.16	0.80	0.29	1.46	0.44	2.20	0.67	3.40	2.30	11.60
Ie4	*2'R-*	0.10	0.29	0.18	0.54	0.27	0.82	0.40	1.24	1.03	3.14	4.06	12.42
	2'S-	0.05	0.35	0.09	0.65	0.14	0.99	0.21	1.49	0.54	3.78	2.11	14.93
Ie5	*2'R-*	0.32	0.98	0.58	1.78	0.85	2.59	1.11	3.39	1.45	4.41	4.25	12.94
	2'S-	0.26	1.33	0.48	2.42	0.70	3.52	0.91	4.61	1.19	6.00	3.48	17.60
Ie6	*2'R-*	0.40	1.10	0.83	2.26	1.04	2.83	1.21	3.30	1.62	4.42	5.88	16.01
	2'S-	0.23	1.31	0.47	2.69	0.59	3.37	0.69	3.93	0.92	5.25	3.32	19.02
Ie7	*2'R-*	0.33	0.78	0.74	1.77	0.98	2.35	1.23	2.95	1.46	3.49	4.42	10.61
	2'S-	0.21	0.98	0.47	2.21	0.63	2.94	0.79	3.69	0.93	4.38	2.83	13.28
Ie8	*2'R-*	0.12	0.41	0.32	0.81	0.43	1.11	0.75	1.91	1.10	2.82	5.45	13.91
	2'S-	0.16	0.54	0.24	1.08	0.32	1.47	0.56	2.54	0.82	3.74	4.07	18.48
mean	*2'R-*	0.31	0.89	0.57	1.61	0.72	2.03	0.96	2.71	1.28	3.64	4.37	12.33
	2'S-	0.23	1.13	0.40	2.03	0.51	2.58	0.68	3.43	0.90	4.61	3.02	15.52
s.d	*2'R-*	0.18	0.53	0.29	0.84	0.31	0.86	0.34	0.94	0.26	0.72	1.17	2.98
	2'S-	0.14	0.68	0.22	1.05	0.23	1.11	0.26	1.21	0.22	0.92	0.80	3.42

Appendix 9: continued

		conjugated carboxyibuprofen (% of administered dose)											
		0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject		*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Ie1	*2'R-*	0.12	0.35	0.47	1.60	0.47	1.60	0.68	2.31	0.80	2.71	3.02	10.23
	2'S-	0.05	0.46	0.40	1.91	0.40	1.91	0.57	2.77	0.67	3.24	2.52	12.22
Ie2	*2'R-*	0.13	0.20	0.20	0.30	0.23	0.34	0.27	0.41	0.31	0.46	0.61	0.91
	2'S-	0.03	0.33	0.05	0.52	0.06	0.59	0.07	0.71	0.08	0.80	0.16	1.57
Ie3	*2'R-*	0.03	0.05	0.04	0.09	0.07	0.16	0.11	0.24	0.17	0.37	0.57	1.27
	2'S-	0.00	0.09	0.01	0.16	0.01	0.29	0.02	0.44	0.03	0.68	0.10	2.31
Ie4	*2'R-*	0.04	0.08	0.02	0.15	0.09	0.22	0.14	0.35	0.36	0.87	1.41	3.44
	2'S-	0.01	0.12	0.01	0.22	0.02	0.35	0.03	0.52	0.08	1.32	0.28	5.21
Ie5	*2'R-*	0.01	0.14	0.01	0.26	0.03	0.37	0.03	0.49	0.05	0.64	0.14	1.87
	2'S-	0.02	0.21	0.04	0.39	0.06	0.57	0.07	0.74	0.09	0.98	0.26	2.85
Ie6	*2'R-*	0.10	0.26	0.21	0.53	0.26	0.66	0.30	0.77	0.41	1.04	1.49	3.75
	2'S-	0.04	0.36	0.08	0.73	0.10	0.92	0.12	1.08	0.16	1.44	0.56	5.19
Ie7	*2'R-*	0.03	0.01	0.07	0.03	0.09	0.04	0.11	0.04	0.13	0.05	0.39	0.16
	2'S-	0.00	0.05	0.00	0.12	0.01	0.16	0.01	0.19	0.01	0.24	0.02	0.71
Ie8	*2'R-*	0.00	0.08	0.08	0.15	0.11	0.21	0.19	0.35	0.28	0.53	1.40	2.60
	2'S-	0.06	0.11	0.04	0.22	0.05	0.30	0.09	0.52	0.12	0.77	0.62	3.79
mean	*2'R-*	0.06	0.15	0.14	0.39	0.17	0.45	0.23	0.62	0.31	0.83	1.13	3.03
	2'S-	0.03	0.22	0.08	0.53	0.09	0.64	0.12	0.87	0.16	1.18	0.57	4.23
s.d	*2'R-*	0.05	0.12	0.15	0.51	0.15	0.50	0.20	0.71	0.23	0.82	0.92	3.16
	2'S-	0.02	0.15	0.13	0.59	0.13	0.57	0.18	0.81	0.21	0.91	0.82	3.60

		free carboxyibuprofen (% of administered dose)											
		0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 24 hr	
Subject		*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Ie1	*2'R-*	0.40	1.19	0.36	0.88	0.36	0.88	0.52	1.27	0.61	1.49	2.29	5.61
	2'S-	0.33	1.37	0.22	1.03	0.22	1.03	0.32	1.48	0.38	1.74	1.43	6.56
Ie2	*2'R-*	0.43	1.49	0.66	2.28	0.76	2.61	0.92	3.16	1.03	3.55	2.03	7.00
	2'S-	0.42	1.91	0.64	2.91	0.73	3.33	0.88	4.03	0.99	4.53	1.95	8.93
Ie3	*2'R-*	0.09	0.31	0.16	0.53	0.30	0.98	0.45	1.47	0.69	2.27	2.35	7.74
	2'S-	0.09	0.37	0.15	0.64	0.28	1.17	0.42	1.76	0.64	2.72	2.20	9.29
Ie4	*2'R-*	0.06	0.21	0.16	0.39	0.18	0.60	0.26	0.89	0.67	2.27	2.65	8.98
	2'S-	0.04	0.23	0.08	0.43	0.12	0.64	0.18	0.97	0.46	2.46	1.83	9.72
Ie5	*2'R-*	0.31	0.84	0.57	1.52	0.82	2.22	1.08	2.90	1.40	3.77	4.11	11.07
	2'S-	0.24	1.12	0.44	2.03	0.64	2.95	0.84	3.87	1.10	5.02	3.22	14.75
Ie6	*2'R-*	0.30	0.84	0.62	1.73	0.78	2.17	0.91	2.53	1.21	3.38	4.39	12.26
	2'S-	0.19	0.95	0.39	1.96	0.49	2.45	0.57	2.85	0.76	3.81	2.76	13.83
Ie7	*2'R-*	0.30	0.77	0.67	1.74	0.89	2.31	1.12	2.91	1.33	3.44	4.03	10.45
	2'S-	0.21	0.93	0.47	2.09	0.62	2.78	0.78	3.50	0.92	4.14	2.81	12.57
Ie8	*2'R-*	0.12	0.33	0.24	0.66	0.32	0.90	0.56	1.56	0.82	2.29	4.05	11.31
	2'S-	0.10	0.43	0.20	0.86	0.27	1.17	0.47	2.02	0.70	2.97	3.45	14.69
mean	*2'R-*	0.25	0.75	0.43	1.22	0.55	1.58	0.73	2.09	0.97	2.81	3.24	9.30
	2'S-	0.20	0.91	0.32	1.49	0.42	1.94	0.56	2.56	0.74	3.42	2.46	11.29
s.d	*2'R-*	0.14	0.45	0.22	0.69	0.29	0.81	0.32	0.88	0.32	0.83	0.99	2.35
	2'S-	0.13	0.57	0.19	0.87	0.23	1.04	0.26	1.16	0.25	1.13	0.71	3.07

Appendix 10: Demographic details and serum albumin concentrations for the young and elderly volunteers of the flurbiprofen study.

Subject Code	Sex	Age (years)	Weight (kg)	Height (cm)
Flurbiprofen study: Young				
Fy1	female	26	73.0	173
Fy2	male	29	65.0	168
Fy3	male	28	64.0	172
Fy4	female	24	69.0	170
Mean	-	26.8	67.8	170.8
s.d.	-	2.2	4.1	2.2
Flurbiprofen study: Elderly				
Fe1	female	72	54.5	155
Fe2	male	82	74.0	175
Fe3	male	87	75.5	173
Fe4	female	73	59.0	164
Mean	-	78.5	65.8	166.8
s.d.	-	7.2	10.6	9.2

Comparison of the means for weight and height between the young and elderly groups using a T-test for independent samples, revealed no significant age-related differences.

Appendix 11: Serum concentrations (mcg/ml) for (*R*)- and (*S*)-flurbiprofen and (*R*)- and (*S*)-4'-hydroxyflurbiprofen in individual healthy young volunteers following the oral administration of 100 mg racemic flurbiprofen.

	(*R*)-flurbiprofen (mcg/ml)						(*S*)-flurbiprofen (mcg/ml)					
Time (hr)	Fy1	Fy2	Fy3	Fy4	mean	s.d.	Fy1	Fy2	Fy3	Fy4	mean	s.d.
0.50	0.42	3.81	1.56	2.11	1.98	1.41	0.38	3.75	1.53	2.15	1.95	1.40
1.00	5.13	8.47	4.47	4.09	5.54	2.00	4.70	8.20	4.28	4.01	5.30	1.96
1.50	6.05	8.85	7.74	4.64	6.82	1.85	5.40	8.34	7.42	4.52	6.42	1.76
2.00	6.34	8.07	7.21	4.57	6.55	1.50	5.47	7.40	7.51	4.76	6.29	1.38
3.00	6.15	5.95	5.16	4.26	5.38	0.86	5.51	5.36	5.74	4.83	5.36	0.39
4.00	4.84	4.30	4.16	3.87	4.29	0.41	4.70	3.97	4.51	4.50	4.42	0.31
5.00	3.76	3.40	3.04	3.23	3.36	0.31	3.53	3.17	3.48	4.00	3.55	0.34
6.00	2.66	2.53	2.39	2.60	2.55	0.12	2.70	2.54	2.80	3.27	2.83	0.31
7.00	2.25	1.86	1.93	2.03	2.02	0.17	2.43	1.92	2.29	2.65	2.32	0.31
8.00	1.81	1.53	1.59	1.71	1.66	0.12	1.99	1.58	1.92	2.36	1.96	0.32
10.00	1.28	1.10	1.25	1.25	1.22	0.08	1.41	1.10	1.49	2.04	1.51	0.39
12.00	0.88	0.74	0.75	0.94	0.83	0.10	1.00	0.80	0.96	1.54	1.08	0.32
24.00	0.12	0.11	0.15	0.23	0.15	0.05	0.19	0.16	0.23	0.42	0.25	0.12

	(*R*)-4'-hydroxyflurbiprofen (mcg/ml)						(*S*)-4'-hydroxyflurbiprofen (mcg/ml)					
Time (hr)	Fy1	Fy2	Fy3	Fy4	mean	s.d.	Fy1	Fy2	Fy3	Fy4	mean	s.d.
0.50	0.03	0.11	0.05	0.02	0.05	0.04	0.04	0.14	0.09	0.04	0.08	0.05
1.00	0.20	0.36	0.29	0.21	0.27	0.08	0.34	0.45	0.47	0.29	0.39	0.09
1.50	0.34	0.54	0.52	0.27	0.42	0.13	0.50	0.69	0.85	0.37	0.60	0.21
2.00	0.43	0.93	0.59	0.28	0.56	0.28	0.59	1.33	0.95	0.39	0.82	0.41
3.00	0.41	0.76	0.54	0.29	0.50	0.20	0.57	1.06	0.84	0.37	0.71	0.30
4.00	0.43	0.59	0.40	0.25	0.42	0.14	0.49	0.77	0.68	0.32	0.57	0.20
5.00	0.21	0.30	0.16	0.16	0.21	0.07	0.23	0.34	0.25	0.20	0.26	0.06
6.00	0.15	0.20	0.13	0.13	0.15	0.03	0.18	0.23	0.20	0.17	0.20	0.03
7.00	0.14	0.16	0.10	0.11	0.13	0.03	0.18	0.19	0.17	0.14	0.17	0.02
8.00	0.12	0.14	0.08	0.08	0.11	0.03	0.16	0.17	0.15	0.12	0.15	0.02
10.00	0.10	0.10	0.06	0.07	0.08	0.02	0.15	0.13	0.12	0.11	0.13	0.02
12.00	0.07	0.07	0.05	0.05	0.06	0.01	0.14	0.09	0.09	0.08	0.10	0.03
24.00	0.02	0.02	0.02	0.02	0.02	0.00	0.07	0.04	0.05	0.05	0.05	0.01

Appendix 12: Serum concentrations (mcg/ml) for (*R*)- and (*S*)-flurbiprofen and (*R*)- and (*S*)-4'-hydroxyflurbiprofen in individual healthy elderly volunteers following the oral administration of 100 mg racemic flurbiprofen.

	(*R*)-flurbiprofen (mcg/ml)						(*S*)-flurbiprofen (mcg/ml)					
Time (hr)	**Fe1**	**Fe2**	**Fe3**	**Fe4**	**mean**	**s.d.**	**Fe1**	**Fe2**	**Fe3**	**Fe4**	**mean**	**s.d.**
0.50	0.00	0.00	7.46	0.24	1.93	3.69	0.00	0.00	7.15	0.20	1.84	3.54
1.00	0.30	0.34	8.12	4.13	3.22	3.73	0.26	0.30	7.69	4.04	3.07	3.55
1.50	1.13	1.67	7.89	7.51	4.55	3.65	1.07	1.52	7.22	7.12	4.23	3.40
2.00	9.27	3.18	7.36	8.93	7.19	2.80	8.56	2.94	6.69	8.19	6.60	2.57
3.00	10.93	5.57	6.56	6.95	7.50	2.36	10.18	5.12	5.99	6.58	6.97	2.22
4.00	8.74	4.63	4.59	5.02	5.75	2.01	9.27	4.80	4.51	5.25	5.96	2.23
5.00	7.14	4.22	3.98	4.36	4.93	1.48	8.02	4.30	4.02	4.68	5.26	1.86
6.00	5.86	3.56	3.59	3.33	4.09	1.19	6.90	3.79	3.75	3.70	4.54	1.58
7.00	4.70	3.00	3.06	3.14	3.48	0.82	5.67	3.25	3.21	3.44	3.89	1.19
8.00	4.15	2.78	2.95	2.69	3.14	0.68	5.01	3.00	3.00	2.91	3.48	1.02
10.00	3.36	2.03	2.42	2.35	2.54	0.57	4.06	2.21	2.52	2.47	2.82	0.84
12.00	2.88	1.72	1.94	1.81	2.09	0.54	3.66	1.89	2.17	1.96	2.42	0.84
24.00	0.89	0.72	0.72	0.43	0.69	0.19	1.61	0.85	1.03	0.71	1.05	0.40

	(*R*)-4'-hydroxyflurbiprofen (mcg/ml)						(*S*)-4'-hydroxyflurbiprofen (mcg/ml)					
Time (hr)	**Fe1**	**Fe2**	**Fe3**	**Fe4**	**mean**	**s.d.**	**Fe1**	**Fe2**	**Fe3**	**Fe4**	**mean**	**s.d.**
0.50	0.00	0.00	0.14	0.00	0.04	0.07	0.00	0.00	0.18	0.00	0.05	0.09
1.00	0.00	0.00	0.25	0.08	0.08	0.12	0.00	0.00	0.29	0.11	0.10	0.14
1.50	0.05	0.09	0.59	0.28	0.25	0.25	0.08	0.14	0.74	0.38	0.34	0.30
2.00	0.36	0.19	0.61	0.42	0.40	0.17	0.51	0.29	0.77	0.55	0.53	0.20
3.00	0.63	0.35	0.63	0.49	0.53	0.13	0.92	0.52	0.74	0.62	0.70	0.17
4.00	0.71	0.44	0.51	0.42	0.52	0.13	0.93	0.56	0.55	0.48	0.63	0.20
5.00	0.41	0.48	0.47	0.37	0.43	0.05	0.52	0.61	0.48	0.42	0.51	0.08
6.00	0.34	0.24	0.27	0.21	0.27	0.06	0.44	0.27	0.26	0.24	0.30	0.09
7.00	0.27	0.21	0.25	0.18	0.23	0.04	0.37	0.24	0.25	0.21	0.27	0.07
8.00	0.23	0.21	0.24	0.16	0.21	0.04	0.34	0.23	0.24	0.20	0.25	0.06
10.00	0.18	0.17	0.23	0.14	0.18	0.04	0.28	0.20	0.24	0.17	0.22	0.05
12.00	0.16	0.16	0.18	0.12	0.16	0.03	0.26	0.18	0.19	0.16	0.20	0.04
24.00	0.07	0.08	0.08	0.05	0.07	0.01	0.14	0.11	0.10	0.09	0.11	0.02

Appendix 13: Cumulative urinary excretion of flurbiprofen enantiomers in individual healthy young volunteers following the oral administration of 100 mg racemic flurbiprofen.

	total flurbiprofen (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fy1	0.71	0.68	1.40	1.33	2.16	2.05	3.33	3.16	5.15	4.88	6.90	6.54	11.76	11.15	12.82	12.16
Fy2	0.66	0.58	2.31	2.04	3.99	3.53	5.34	4.72	5.80	5.13	6.26	5.54	7.74	6.85	8.50	7.52
Fy3	0.21	0.17	2.09	1.70	3.95	3.21	5.23	4.25	6.01	4.89	6.55	5.32	10.47	8.52	10.65	8.66
Fy4	0.29	0.24	0.41	0.34	1.31	1.08	3.73	3.08	5.14	4.25	7.37	6.09	13.12	10.85	13.73	11.35
mean	0.47	0.42	1.55	1.35	2.85	2.47	4.41	3.80	5.53	4.79	6.77	5.87	10.77	9.34	11.42	9.92
s.d.	0.26	0.25	0.86	0.74	1.34	1.12	1.03	0.81	0.45	0.38	0.48	0.55	2.29	2.04	2.34	2.19

	conjugated flurbiprofen (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fy1	0.43	0.41	0.85	0.80	1.31	1.23	2.01	1.90	3.12	2.95	4.17	3.95	7.12	6.73	7.76	7.34
Fy2	0.54	0.48	1.90	1.69	3.29	2.92	4.40	3.91	4.79	4.25	5.16	4.58	6.38	5.66	7.01	6.22
Fy3	0.16	0.13	1.65	1.34	3.12	2.53	4.14	3.35	4.76	3.85	5.18	4.19	8.29	6.71	8.43	6.82
Fy4	0.22	0.18	0.31	0.26	1.00	0.82	2.86	2.35	3.94	3.23	5.65	4.64	10.06	8.26	10.53	8.64
mean	0.34	0.30	1.18	1.02	2.18	1.88	3.35	2.88	4.15	3.57	5.04	4.34	7.96	6.84	8.43	7.26
s.d.	0.18	0.17	0.73	0.63	1.19	1.01	1.12	0.91	0.79	0.59	0.62	0.33	1.61	1.07	1.51	1.03

	free flurbiprofen (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fy1	0.28	0.27	0.55	0.53	0.85	0.81	1.31	1.25	2.03	1.94	2.72	2.59	4.64	4.42	5.06	4.82
Fy2	0.12	0.10	0.40	0.35	0.70	0.61	0.94	0.82	1.02	0.89	1.10	0.96	1.36	1.18	1.49	1.30
Fy3	0.04	0.04	0.43	0.36	0.82	0.68	1.09	0.90	1.25	1.04	1.36	1.13	2.18	1.81	2.22	1.84
Fy4	0.07	0.06	0.09	0.08	0.30	0.26	0.87	0.74	1.20	1.01	1.72	1.45	3.06	2.59	3.20	2.71
mean	0.13	0.12	0.37	0.33	0.67	0.59	1.05	0.93	1.38	1.22	1.73	1.53	2.81	2.50	2.99	2.67
s.d.	0.11	0.11	0.20	0.18	0.25	0.24	0.20	0.23	0.45	0.48	0.71	0.74	1.40	1.40	1.55	1.55

Appendix 14: Cumulative urinary excretion of 4'-hydroxyflurbiprofen enantiomers in individual healthy young volunteers following the oral administration of 100 mg racemic flurbiprofen.

	total 4'-hydroxyflurbiprofen (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fy1	1.44	1.18	2.82	2.31	4.35	3.56	6.70	5.49	10.37	8.50	13.89	11.39	23.69	19.41	25.83	21.17
Fy2	2.07	1.72	7.24	6.01	12.53	10.39	16.74	13.88	18.20	15.10	19.63	16.28	24.27	20.13	26.66	22.11
Fy3	0.45	0.40	4.56	4.05	8.62	7.67	11.42	10.16	13.13	11.68	14.31	12.73	22.88	20.35	23.27	20.70
Fy4	0.54	0.46	0.76	0.65	2.43	2.08	6.94	5.95	9.56	8.19	13.71	11.74	24.41	20.91	25.55	21.88
mean	1.12	0.94	3.84	3.25	6.98	5.93	10.45	8.87	12.82	10.87	15.39	13.03	23.81	20.20	25.33	21.46
s.d.	0.77	0.63	2.75	2.30	4.51	3.80	4.72	3.95	3.90	3.23	2.84	2.24	0.70	0.62	1.45	0.65

	4'-hydroxyflurbiprofen acyl-conjugate (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fy1	0.87	0.71	1.71	1.38	2.63	2.14	4.06	3.30	6.29	5.10	8.42	6.83	14.36	11.65	15.66	12.70
Fy2	1.52	1.25	5.33	4.36	9.21	7.55	12.31	10.08	13.38	10.96	14.43	11.82	17.84	14.62	19.60	16.06
Fy3	0.35	0.30	3.52	3.01	6.65	5.69	8.81	7.54	10.14	8.67	11.04	9.44	17.66	15.10	17.96	15.36
Fy4	0.41	0.34	0.58	0.48	1.85	1.53	5.29	4.37	7.29	6.02	10.46	8.64	18.61	15.37	19.48	16.09
mean	0.79	0.65	2.78	2.31	5.09	4.23	7.62	6.32	9.28	7.69	11.09	9.18	17.12	14.19	18.18	15.05
s.d.	0.54	0.44	2.08	1.72	3.46	2.87	3.72	3.09	3.19	2.66	2.50	2.07	1.88	1.72	1.84	1.60

	4'-hydroxyflurbiprofen phenol-conjugate (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fy1	0.04	0.00	0.09	0.00	0.13	0.00	0.20	0.00	0.31	0.00	0.42	0.00	0.72	0.00	0.78	0.00
Fy2	0.26	0.21	0.90	0.73	1.55	1.27	2.07	1.70	2.25	1.84	2.43	1.99	3.00	2.46	3.30	2.70
Fy3	0.01	0.02	0.14	0.16	0.26	0.31	0.34	0.41	0.39	0.47	0.42	0.51	0.68	0.82	0.69	0.83
Fy4	0.01	0.01	0.01	0.02	0.04	0.05	0.12	0.14	0.16	0.19	0.23	0.27	0.41	0.49	0.43	0.51
mean	0.08	0.06	0.28	0.23	0.49	0.41	0.68	0.56	0.78	0.63	0.88	0.69	1.20	0.94	1.30	1.01
s.d.	0.12	0.10	0.41	0.35	0.71	0.59	0.93	0.78	0.99	0.83	1.04	0.89	1.21	1.07	1.34	1.18

	free 4'-hydroxyflurbiprofen (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fy1	0.52	0.47	1.02	0.92	1.58	1.43	2.44	2.20	3.78	3.40	5.06	4.56	8.62	7.77	9.40	8.47
Fy2	0.29	0.26	1.02	0.91	1.77	1.58	2.37	2.11	2.57	2.29	2.78	2.47	3.43	3.06	3.77	3.36
Fy3	0.09	0.09	0.91	0.88	1.71	1.67	2.27	2.21	2.61	2.54	2.84	2.77	4.54	4.42	4.62	4.50
Fy4	0.12	0.11	0.17	0.16	0.54	0.50	1.53	1.44	2.11	1.98	3.03	2.83	5.39	5.05	5.64	5.28
mean	0.26	0.23	0.78	0.72	1.40	1.29	2.15	1.99	2.77	2.55	3.42	3.16	5.50	5.07	5.86	5.40
s.d.	0.20	0.18	0.41	0.38	0.58	0.54	0.42	0.37	0.71	0.61	1.09	0.95	2.23	1.98	2.48	2.19

Appendix 15: Cumulative urinary excretion of 3'-hydroxy-4'-methoxyflurbiprofen enantiomers in individual healthy young volunteers following the oral administration of 100 mg racemic flurbiprofen.

	total 3'-hydroxy-4'-methoxyflurbiprofen (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fy1	0.13	0.13	0.25	0.26	0.39	0.40	0.59	0.62	0.92	0.96	1.23	1.29	2.10	2.20	2.29	2.40
Fy2	0.13	0.20	0.47	0.70	0.81	1.22	1.08	1.63	1.18	1.77	1.27	1.91	1.57	2.36	1.73	2.59
Fy3	0.04	0.04	0.40	0.44	0.75	0.83	0.99	1.10	1.14	1.26	1.24	1.38	1.98	2.20	2.02	2.24
Fy4	0.03	0.04	0.05	0.05	0.15	0.17	0.44	0.47	0.61	0.65	0.87	0.93	1.55	1.66	1.63	1.74
mean	0.08	0.10	0.29	0.36	0.52	0.65	0.78	0.96	0.96	1.16	1.15	1.38	1.80	2.11	1.92	2.24
s.d.	0.05	0.08	0.19	0.28	0.31	0.47	0.31	0.52	0.26	0.48	0.19	0.40	0.28	0.30	0.30	0.37

	3'-hydroxy-4'-methoxyflurbiprofen acyl-conjugate (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fy1	0.003	0.002	0.006	0.004	0.009	0.006	0.014	0.009	0.021	0.013	0.029	0.018	0.049	0.030	0.053	0.033
Fy2	0.005	0.004	0.017	0.015	0.030	0.026	0.040	0.035	0.044	0.038	0.047	0.041	0.058	0.051	0.064	0.056
Fy3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Fy4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
mean	0.002	0.002	0.006	0.005	0.010	0.008	0.013	0.011	0.016	0.013	0.019	0.015	0.027	0.020	0.029	0.022
s.d.	0.002	0.002	0.008	0.007	0.014	0.013	0.019	0.017	0.021	0.018	0.023	0.020	0.031	0.025	0.034	0.027

	3'-hydroxy-4'-methoxyflurbiprofen phenol-conjugate (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fy1	0.12	0.13	0.24	0.25	0.37	0.39	0.56	0.59	0.87	0.92	1.17	1.23	1.99	2.10	2.17	2.29
Fy2	0.13	0.20	0.45	0.68	0.77	1.18	1.03	1.58	1.12	1.72	1.21	1.86	1.50	2.29	1.65	2.52
Fy3	0.04	0.04	0.40	0.44	0.75	0.83	0.99	1.10	1.14	1.26	1.24	1.38	1.98	2.20	2.02	2.24
Fy4	0.03	0.04	0.05	0.05	0.15	0.17	0.44	0.47	0.61	0.65	0.87	0.93	1.55	1.66	1.63	1.74
mean	0.08	0.10	0.28	0.36	0.51	0.64	0.76	0.94	0.94	1.14	1.12	1.35	1.76	2.06	1.87	2.20
s.d.	0.05	0.08	0.18	0.27	0.30	0.46	0.30	0.51	0.25	0.46	0.17	0.38	0.27	0.28	0.27	0.33

	free 3'-hydroxy-4'-methoxyflurbiprofen (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fy1	0.004	0.004	0.007	0.009	0.011	0.013	0.017	0.020	0.026	0.032	0.034	0.042	0.059	0.072	0.064	0.079
Fy2	0.001	0.001	0.005	0.005	0.008	0.008	0.011	0.011	0.012	0.012	0.013	0.013	0.016	0.016	0.018	0.018
Fy3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Fy4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
mean	0.001	0.001	0.003	0.003	0.005	0.005	0.007	0.008	0.009	0.011	0.012	0.014	0.019	0.022	0.021	0.024
s.d.	0.002	0.002	0.004	0.004	0.006	0.007	0.008	0.010	0.012	0.015	0.016	0.020	0.028	0.034	0.030	0.037

Appendix 16: Cumulative urinary excretion of flurbiprofen enantiomers in individual healthy elderly volunteers following the oral administration of 100 mg racemic flurbiprofen.

	total flurbiprofen (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fe1	2.15	1.64	2.46	1.88	3.04	2.32	3.41	2.60	4.13	3.15	4.77	3.64	14.65	11.16	17.15	13.07
Fe2	1.13	0.85	1.34	1.01	1.72	1.30	2.38	1.80	3.04	2.30	3.19	2.41	7.24	5.46	7.84	5.91
Fe3	0.19	0.17	0.49	0.42	0.77	0.67	1.02	0.88	1.52	1.31	2.17	1.87	7.91	6.81	9.29	8.00
Fe4	1.70	1.46	2.48	2.13	3.54	3.04	4.38	3.76	5.69	4.89	6.31	5.43	13.04	11.21	13.55	11.65
mean	1.30	1.03	1.69	1.36	2.27	1.83	2.80	2.26	3.60	2.91	4.11	3.33	10.71	8.66	11.96	9.66
s.d.	0.85	0.67	0.96	0.79	1.26	1.06	1.44	1.23	1.76	1.52	1.82	1.58	3.69	2.97	4.23	3.29

	conjugated flurbiprofen (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fe1	1.30	1.03	1.49	1.18	1.84	1.45	2.06	1.63	2.50	1.97	2.88	2.28	8.85	7.00	10.36	8.20
Fe2	0.49	0.38	0.58	0.45	0.75	0.58	1.04	0.81	1.32	1.03	1.39	1.08	3.15	2.46	3.41	2.66
Fe3	0.02	0.02	0.05	0.04	0.08	0.06	0.10	0.09	0.15	0.13	0.21	0.18	0.78	0.66	0.92	0.78
Fe4	1.18	1.05	1.72	1.53	2.46	2.18	3.04	2.69	3.95	3.50	4.39	3.88	9.07	8.03	9.42	8.34
mean	0.75	0.62	0.96	0.80	1.28	1.07	1.56	1.30	1.98	1.66	2.22	1.86	5.46	4.54	6.03	5.00
s.d.	0.60	0.51	0.78	0.67	1.07	0.94	1.27	1.12	1.63	1.44	1.81	1.60	4.15	3.54	4.59	3.86

	free flurbiprofen (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fe1	0.85	0.61	0.98	0.70	1.20	0.86	1.35	0.97	1.64	1.17	1.89	1.36	5.80	4.16	6.79	4.87
Fe2	0.64	0.47	0.76	0.55	0.97	0.71	1.35	0.99	1.72	1.26	1.80	1.32	4.09	3.00	4.43	3.25
Fe3	0.17	0.15	0.44	0.38	0.70	0.60	0.92	0.79	1.37	1.18	1.95	1.68	7.13	6.15	8.37	7.22
Fe4	0.52	0.42	0.76	0.61	1.08	0.87	1.33	1.07	1.73	1.39	1.92	1.54	3.97	3.19	4.13	3.31
mean	0.55	0.41	0.73	0.56	0.99	0.76	1.24	0.95	1.62	1.25	1.89	1.48	5.25	4.12	5.93	4.66
s.d.	0.28	0.19	0.22	0.13	0.22	0.13	0.21	0.12	0.17	0.10	0.06	0.17	1.51	1.44	2.02	1.86

Appendix 17: Cumulative urinary excretion of 4'-hydroxyflurbiprofen enantiomers in individual healthy elderly volunteers following the oral administration of 100 mg racemic flurbiprofen.

	total 4'-hydroxyflurbiprofen (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fe1	2.50	1.99	2.86	2.28	3.53	2.81	3.96	3.16	4.79	3.82	5.53	4.41	16.99	13.55	19.89	15.86
Fe2	2.51	2.27	2.97	2.68	3.82	3.45	5.28	4.77	6.75	6.10	7.08	6.40	16.04	14.50	17.38	15.71
Fe3	0.37	0.28	0.94	0.72	1.49	1.13	1.96	1.49	2.93	2.22	4.17	3.16	15.22	11.54	17.88	13.56
Fe4	3.12	2.36	4.54	3.44	6.49	4.91	8.02	6.07	10.42	7.88	11.56	8.75	23.90	18.07	24.83	18.78
mean	2.12	1.73	2.83	2.28	3.83	3.07	4.81	3.87	6.22	5.01	7.09	5.68	18.04	14.42	19.99	15.98
s.d.	1.20	0.97	1.47	1.15	2.05	1.57	2.54	1.99	3.20	2.49	3.21	2.44	3.97	2.73	3.40	2.14

	4'-hydroxyflurbiprofen acyl-conjugate (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fe1	0.80	0.57	0.91	0.65	1.12	0.80	1.26	0.90	1.52	1.09	1.76	1.26	5.41	3.88	6.33	4.54
Fe2	1.45	1.29	1.71	1.52	2.20	1.96	3.05	2.71	3.90	3.46	4.08	3.63	9.26	8.22	10.03	8.91
Fe3	0.33	0.24	0.83	0.61	1.32	0.97	1.74	1.27	2.59	1.90	3.69	2.71	13.47	9.88	15.82	11.61
Fe4	0.85	0.58	1.24	0.85	1.77	1.21	2.18	1.50	2.84	1.94	3.15	2.16	6.51	4.46	6.76	4.63
mean	0.86	0.67	1.17	0.91	1.60	1.23	2.06	1.60	2.71	2.10	3.17	2.44	8.66	6.61	9.73	7.42
s.d.	0.46	0.44	0.40	0.42	0.48	0.51	0.76	0.78	0.97	0.99	1.02	0.99	3.59	2.91	4.38	3.46

	4'-hydroxyflurbiprofen phenol-conjugate (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fe1	0.28	0.19	0.32	0.22	0.39	0.27	0.44	0.31	0.53	0.37	0.61	0.43	1.88	1.32	2.20	1.55
Fe2	0.03	0.04	0.04	0.05	0.05	0.06	0.07	0.09	0.09	0.11	0.10	0.11	0.22	0.26	0.24	0.28
Fe3	0.00	0.01	0.01	0.02	0.01	0.04	0.01	0.05	0.02	0.07	0.03	0.10	0.11	0.36	0.13	0.42
Fe4	0.20	0.09	0.29	0.12	0.41	0.18	0.50	0.22	0.65	0.29	0.73	0.32	1.50	0.65	1.56	0.68
mean	0.13	0.08	0.16	0.10	0.22	0.14	0.26	0.16	0.32	0.21	0.37	0.24	0.93	0.65	1.03	0.73
s.d.	0.13	0.08	0.16	0.09	0.21	0.11	0.25	0.12	0.31	0.14	0.35	0.16	0.89	0.48	1.01	0.57

	free 4'-hydroxyflurbiprofen (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fe1	1.43	1.23	1.63	1.40	2.01	1.73	2.26	1.94	2.74	2.35	3.16	2.72	9.70	8.34	11.36	9.76
Fe2	1.03	0.94	1.22	1.11	1.56	1.43	2.16	1.98	2.77	2.53	2.90	2.65	6.57	6.02	7.12	6.52
Fe3	0.04	0.03	0.10	0.08	0.16	0.13	0.21	0.17	0.32	0.25	0.45	0.36	1.64	1.30	1.93	1.53
Fe4	2.07	1.69	3.02	2.46	4.32	3.52	5.33	4.35	6.93	5.65	7.69	6.27	15.89	12.96	16.51	13.47
mean	1.14	0.97	1.49	1.26	2.01	1.70	2.49	2.11	3.19	2.70	3.55	3.00	8.45	7.16	9.23	7.82
s.d.	0.85	0.70	1.21	0.98	1.73	1.40	2.12	1.72	2.75	2.23	3.02	2.44	5.97	4.85	6.20	5.07

Appendix 18: Cumulative urinary excretion of 3'-hydroxy-4'-methoxyflurbiprofen enantiomers in individual healthy elderly volunteers following the oral administration of 100 mg racemic flurbiprofen.

	total 3'-hydroxy-4'-methoxyflurbiprofen (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fe1	0.17	0.14	0.19	0.16	0.24	0.20	0.27	0.23	0.32	0.27	0.37	0.32	1.15	0.97	1.34	1.14
Fe2	0.25	0.22	0.30	0.26	0.39	0.33	0.54	0.45	0.68	0.58	0.72	0.61	1.63	1.38	1.76	1.49
Fe3	0.04	0.04	0.11	0.10	0.17	0.15	0.22	0.20	0.33	0.30	0.47	0.42	1.71	1.53	2.01	1.80
Fe4	0.13	0.15	0.19	0.21	0.27	0.30	0.33	0.38	0.43	0.49	0.48	0.54	0.98	1.12	1.02	1.16
mean	0.15	0.14	0.20	0.18	0.26	0.25	0.34	0.31	0.44	0.41	0.51	0.47	1.37	1.25	1.53	1.40
s.d.	0.09	0.07	0.08	0.07	0.09	0.08	0.14	0.12	0.17	0.15	0.15	0.13	0.36	0.25	0.44	0.31

	3'-hydroxy-4'-methoxyflurbiprofen acyl-conjugate (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fe1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Fe2	0.006	0.005	0.007	0.006	0.009	0.008	0.013	0.011	0.017	0.014	0.018	0.015	0.040	0.033	0.043	0.036
Fe3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Fe4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
mean	0.002	0.001	0.002	0.002	0.002	0.002	0.003	0.003	0.004	0.003	0.004	0.004	0.010	0.008	0.011	0.009
s.d.	0.003	0.003	0.004	0.003	0.005	0.004	0.007	0.005	0.008	0.007	0.009	0.007	0.020	0.017	0.022	0.018

	3'-hydroxy-4'-methoxyflurbiprofen phenol-conjugate (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fe1	0.17	0.14	0.19	0.16	0.24	0.20	0.27	0.23	0.32	0.27	0.37	0.32	1.15	0.97	1.34	1.14
Fe2	0.24	0.20	0.28	0.24	0.36	0.31	0.50	0.43	0.64	0.55	0.67	0.57	1.53	1.30	1.66	1.41
Fe3	0.04	0.04	0.11	0.10	0.17	0.15	0.22	0.20	0.33	0.30	0.47	0.42	1.71	1.53	2.01	1.80
Fe4	0.13	0.15	0.19	0.21	0.27	0.30	0.33	0.38	0.43	0.49	0.48	0.54	0.98	1.12	1.02	1.16
mean	0.14	0.13	0.19	0.18	0.26	0.24	0.33	0.31	0.43	0.40	0.50	0.46	1.34	1.23	1.51	1.38
s.d.	0.08	0.07	0.07	0.06	0.08	0.08	0.12	0.11	0.15	0.14	0.13	0.12	0.34	0.24	0.42	0.31

	free 3'-hydroxy-4'-methoxyflurbiprofen (% of administered dose)															
	0-2 hr		0 -4 hr		0 - 6 hr		0 - 8 hr		0 - 10 hr		0 - 12 hr		0 - 22 hr		0 - 24 hr	
Subject	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Fe1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Fe2	0.009	0.008	0.011	0.009	0.014	0.012	0.019	0.016	0.024	0.021	0.025	0.022	0.057	0.050	0.062	0.054
Fe3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Fe4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
mean	0.002	0.002	0.003	0.002	0.003	0.003	0.005	0.004	0.006	0.005	0.006	0.005	0.014	0.012	0.016	0.013
s.d.	0.004	0.004	0.005	0.005	0.007	0.006	0.009	0.008	0.012	0.010	0.013	0.011	0.029	0.025	0.031	0.027